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THE FIXATION OF CARBON DIOXIDE IN OXALACETIC ACID AND ITS RELATIONSHIP TO BACTERIAL RESPIRATION

by

Lester Orville Krampitz

A Thesis Submitted to the Graduate Faculty
for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject: Physiological Bacteriology

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INTRODUCTION

The active participation of carbon dioxide in heterotrophic metabolism was considered only a remote possibility prior to 1935. In that year Wood and Werkman (1935) advanced the principle of heterotrophic assimilation of carbon dioxide as a definite and experimentally supported concept. These investigators found with several species of Propionibacterium that the total carbon dioxide liberated during fermentation of glycerol plus that remaining in the form of carbonate was less than the original carbon dioxide added as carbonate. Their initial proposal was not readily accepted owing perhaps in large measure, to the unexpected nature of the concept. The continued enthusiasm of these workers maintained the interest and now investigators throughout the world have accepted the principle, and are making notable contributions to the mechanisms involved in carbon dioxide utilization.

The most natural consequence of the investigations which followed was an attempt to elucidate the mechanism by which carbon dioxide enters metabolic processes. Wood and Werkman (1938) obtained data which led them to the hypothesis that a three carbon compound condensed with carbon dioxide

yielding a four carbon compound. This they referred to as the C_3 and C_1 addition hypothesis, wherein a carbon to carbon linkage is formed. Later they proposed pyruvic acid as the three carbon constituent which after condensation with carbon dioxide yields oxalacetic acid. The present investigation is concerned with the isolation of the specific enzyme which is responsible for the fixation reaction and with the characterization of this enzyme. In addition, evidence that has been obtained with the aid of the stable isotope of carbon (C^{13}), will be presented to prove that fixation is brought about by this enzyme.

HISTORICAL

A clear definition of heterotrophic assimilation of carbon dioxide is desirable before the historical is developed. The participation of this relatively inert substance to synthesize materials by autotrophic forms of life, both photosynthetic and chemosynthetic, has been known; however, the mechanism involved is as yet unsolved. There has been a tendency by certain investigators to confuse heterotrophic assimilation with autotrophic assimilation. While no sharp distinction can be made between the two forms, Werkman and Wood (1942a) have differentiated

the two from the standpoint of mechanism as follows: " . . . the heterotroph can bring about a carbon to carbon linkage when one component of the linkage is organic, but it cannot repeat the process to form a linkage in which both components originate from inorganic carbon. In the case of the autotroph this can be done, however, adjacent carbons can be inorganic." Clearly a reduction of carbon dioxide to formic acid, a reaction demonstrated by Woods (1936), is not a synthetic reaction in the sense that a carbon to carbon linkage is formed. The importance of this reaction may in the future be decided, if and when it is shown that formic acid, by further synthetic reactions may produce complex cellular substances. The heterotrophic synthesis of organic compounds involving inorganic carbon is the only type that will be considered in this thesis. Reference to direct carbon dioxide reductions by heterotrophic forms will be made in order that subsequent confusion will be eliminated. These reductions include the already mentioned reduction of carbon dioxide to formic acid by the coliform bacteria (Woods, 1936). Barker et al. (1940a) isolated the organism Clostridium acidi-urici which attacks purine compounds anaerobically to form cell substance, ammonia, acetic acid and carbon dioxide. By use of radioactive carbon dioxide it was shown that the acetic acid was synthesized from carbon dioxide, and that the fixed carbon occurred in both the methyl and carboxyl

groups. This organism may be an example of a heterotroph forming a carbon chain from C_1 compounds, i.e., an autotrophic property. The formation of methane by the reduction of carbon dioxide with ethyl alcohol as the reductant has been shown by Barker (1936) with Methanobacterium omelianskii. Likewise, the reduction of carbon dioxide to methane by acetic acid oxidation has been proposed by Barker et al. (1940b).

Wood and Werkman (1935) reported the utilization of carbon dioxide by several species of Propionibacterium during the dissimilation of glycerol. In 1938 these same authors showed an equimolar relationship between the carbon dioxide fixed and the succinic acid formed. In 1940 they found that the inhibition of fixation by sodium fluoride resulted in a corresponding reduction in succinic acid. From this information they proposed the C_3 and C_1 addition hypothesis to account for the fixation of carbon dioxide. Pyruvic acid was suggested as the possible C_3 constituent since it could be isolated from the fermentation. Empirically the equation became:

$CH_3.CO.COOH + CO_2 \longrightarrow COOH.CH_2.CO.COOH$

The formation of succinate results from the stepwise reduction of oxalacetate through malate and fumarate. Phelps et al. (1939) confirmed the work of Wood and Werkman.

In the case of Propionibacterium the demonstration of

carbon dioxide assimilation is not as difficult as with other genera for the reason that there is a net uptake of carbon dioxide, i.e., the quantity utilized is greater than the amount produced during the dissimilation of glycerol. With most heterotrophic genera, however the net production of carbon dioxide is greater than that consumed, consequently most of the evidence was indirect. Thus the observation of Elsden (1938) that the rate of succinic acid formation by Escherichia coli was a function of the concentration of carbon dioxide in the medium constituted indirect evidence of carbon dioxide assimilation.

The availability of the isotopes of carbon, the radioactive C^{11} and the stable isotope C^{13}, has greatly facilitated further investigation. Fixation by the propionic acid bacteria fermenting glycerol was confirmed by Carson and Ruben (1940) using radioactive carbon and by Wood et al. (1940) employing the stable isotope. The fixed carbon was found in the carboxyl groups of the succinic and propionic acids. These results, although they suggested the C_3 and C_1 hypothesis, were not rigid proof because the isotopic carbon was found in both the propionic acid and in the succinic acid in contrast to the predictions made on the basis of the stoichiometric relationship between carbon dioxide fixed and the succinic acid formed.

Wood et al. (1941a) also demonstrated the fixation of

carbon dioxide in the coliform bacteria during the fermentation of galactose, pyruvate and citrate. In this case the fixed isotope occurred only in the succinic and formic acids. The latter was formed by the reduction of carbon dioxide and the former probably by the C_3 and C_1 hypothesis.

Slade et al. (1942) used $C^{13}O_2$ to investigate fixation by several heterotrophic genera namely, Staphylococcus, Aerobacter, Streptococcus, Clostridium, Proteus and Lactobacillus. In most cases glucose and citric acid were used as the substrates, and in all species forming succinate, fixed carbon was found exclusively in the carboxyl groups of this acid. The quantitative data suggest that only one carboxyl group contained the fixed carbon which again supports the C_3 and C_1 hypothesis. Fixed carbon was also contained in the carboxyl group of lactic acid from several species. The authors attempted to explain this occurrence by C_3 and C_1 addition, and furthermore have suggested other mechanisms. Experiments performed with the homo-lactic acid bacteria showed no fixation. With some genera, Aerobacter and Clostridium, acetic acid with fixed carbon in the carboxyl group was isolated. The mechanism of this fixation is unknown, however it may have been formed by the cleavage of a C_4 dicarboxylic acid.

Nishima, Endo and Nakayama (1941) by use of radioactive carbon have demonstrated the synthesis of malic and fumaric

acids from pyruvic acid and carbon dioxide in fermentations by Escherichia coli. Wood and Werkman (1938) proposed that the oxalacetate formed by pyruvate and carbon dioxide condensation was stepwise reduced to malate, fumarate and succinate. The occurrence of the isotope in the intermediate stages of reduction tends to validate the Wood and Werkman reaction.

It is clear from the investigations mentioned that many heterotrophic forms utilize carbon dioxide. In the words of Werkman and Wood (1942a), " The question is not so much whether it occurs but how it occurs." The evidence points to C_3 and C_1 addition but it must be emphasized that the actual fixation reaction has not been demonstrated.

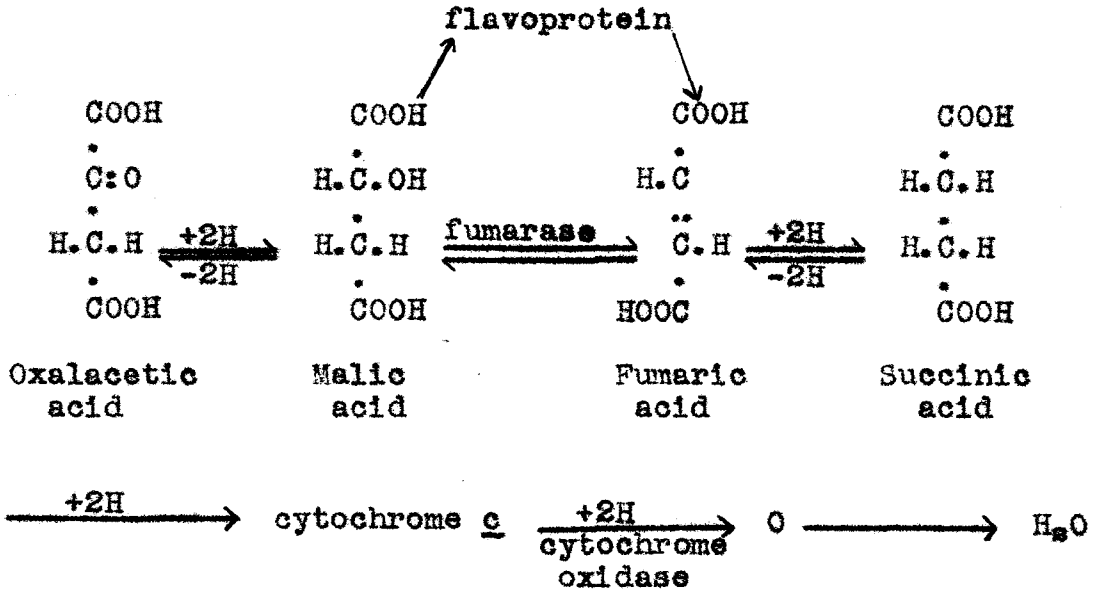
Krebs and Eggleston (1940a) (1941) in two articles entitled, The Biological Synthesis of Oxalacetate from Pyruvic Acid and Carbon Dioxide, imply that they show carbon dioxide fixation with pyruvic acid to form oxalacetic acid. In no case was the synthesis of oxalacetate shown; the fixation of carbon dioxide was not demonstrated and in one investigation pyruvic acid was not used as the substrate. They did show that carbon dioxide stimulated succinic acid formation from pyruvate by pigeon liver.

This fixation of carbon dioxide by pigeon liver was the first demonstration that this unique process occurs in some mammalian tissues. In order to clarify the steps involved

in mammalian tissue respiration and pyruvic acid dissimilation to carbon dioxide and water, and to correlate these steps with carbon dioxide fixation by the C_3 and C_4 addition hypothesis, the classical researches of the Szent-Györgyi school and Krebs and his co-workers will be cited.

Szent-Gyorgyi and his co-workers (1937) proposed what is known as the Szent-Györgyi scheme of hydrogen transport.

The details of the scheme are outlined below:



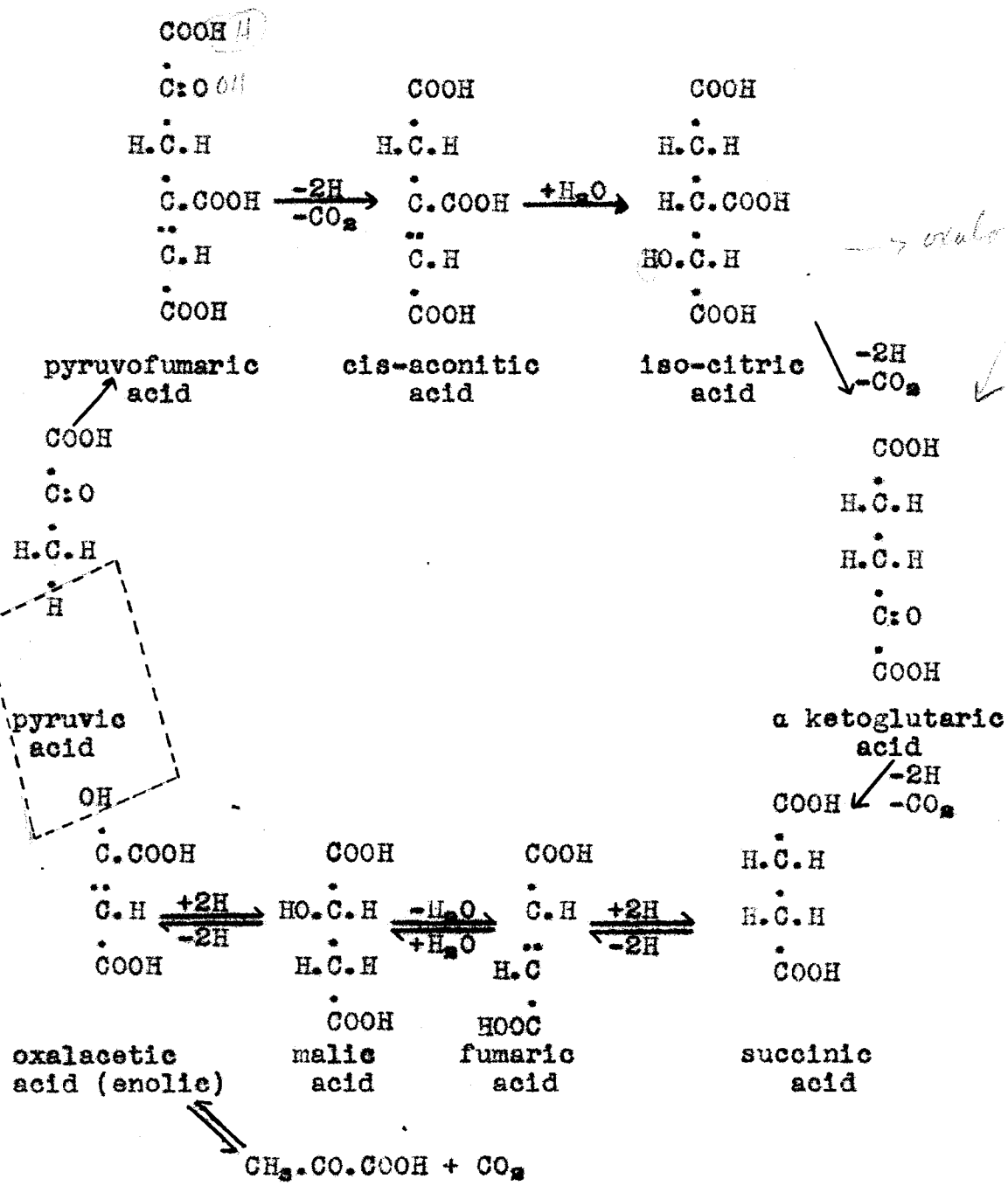
The C_4 dicarboxylic acids act as hydrogen transporters in the following manner: Oxalacetic acid accepts hydrogen from some oxidizable substrate. A typical example is the oxidation of the triose phosphate, an intermediate in the Embden-Meyerhof-Parnas scheme of carbohydrate dissimilation. The oxalacetate on reduction becomes malic acid which transfers its hydrogen to flavoprotein, the malic acid returns to the oxidized state, namely oxalacetic acid. The reduced flavo-

protein passes the hydrogen to fumaric acid which becomes succinic acid. The latter transfers its hydrogen to oxygen via cytochrome c and cytochrome oxidase. By this continual oxidation and reduction of these C_4 dicarboxylic acids, hydrogen is removed from the original reduced substrate. Obviously for these oxidations and reductions to take place the protein moiety of the enzymes must be present; these acids act as coenzymes and this point should be emphasized --- the acids are not intermediates of carbohydrate dissimilation, and the mechanism explains only the pathway by which the hydrogen ultimately reaches its oxidant. It is obvious that a constant supply of C_4 acids must be maintained. The contention presented here is that this maintenance occurs by the carboxylation of pyruvic acid.

Krebs and Johnson (1937) proposed the citric acid cycle as a mechanism for the oxidation of pyruvic acid in mammalian muscle tissue. This cycle explains the complete oxidation of pyruvic acid, not merely the pathway for the transport of hydrogen but also the elimination of the carbon atoms in their highest state of oxidation as carbon dioxide. The cycle is diagrammed on the following page.

The cycle begins with the condensation of pyruvic acid with oxalacetic acid, and passes through a series of oxidative decarboxylations until the end-product is oxalacetic

Krebs' Cycle



Wood and Werkman reaction

acid again. The final result is the elimination of the three carbon atoms and the hydrogen of the pyruvic acid, while the oxalacetic acid has been regenerated. It will be observed that the last four steps of the cycle are reversible and include components of the Szent-Györgyi cycle, but here they represent intermediary compounds of pyruvate dissimilation as well as hydrogen transporters. If any of these C_4 acids are used as hydrogen carriers, the cycle is broken and the need for more oxalacetate becomes acute. Wood and Werkman (1938) suggested that oxalacetic acid is synthesized by the carbon dioxide utilization reaction in muscle tissue.

The Krebs cycle has been shown to function in pigeon breast muscle and liver tissue, however one important observation was made which indicates that the two tissues are not identical in their dissimilatory mechanisms. Malonic acid, owing to its close structural relationship to succinic acid, inhibits the succinic dehydrogenase. This enzyme activates succinate for the oxidation to fumarate. When malonate is placed in the presence of pigeon breast muscle and pyruvate, the dissimilation of the latter is stopped (Krebs and Eggleston, 1940b). Upon the addition of fumaric or malic acids the dissimilation of pyruvate will proceed proportionally to the amount of C_4 acid added. Inhibition of the succinate oxidation stops the regeneration of oxalacetate which is necessary for the cycle to operate. Addition of fumarate

or malate relieves this inhibition owing to the interconversion of these acids to oxalacetic acid. Evans (1940) found that pyruvate dissimilation in pigeon liver is not sensitive to malonic acid inhibition. However, many of the intermediates of the Krebs cycle could be demonstrated, mainly the C_4 acids and α ketoglutaric acid. When the oxidation in liver tissue was compared to that in muscle tissue, it became evident that the liver tissue was capable of synthesizing its oxalacetate by some other mechanism. The only malonate-insensitive reaction that has been described in the literature for synthesis of C_4 dicarboxylic acids is the fixation reaction of Wood and Werkman. Independently, Evans and Slotin (1940) and Krebs and Eggleston (1940a) and shortly afterward Wood et al. (1941b) presented evidence that carbon dioxide is fixed by C_3 and C_1 addition in pigeon liver. Evans and Slotin (1940) showed that carbon dioxide is fixed during the oxidation of pyruvic acid by the isolation of radioactive α ketoglutarate from experiments in which $C^{11}O_2$ was used as a tracer. Wood et al. (1941b) employed heavy carbon and Evans and Slotin (1941) radioactive carbon; both groups showed the position of the carbon fixed in isolated α ketoglutaric acid to be in the carboxyl group adjacent to the carbonyl group. The original Krebs cycle postulated citric acid as an intermediate. The reactions citrate $\rightleftharpoons$ cis aconitate $\rightleftharpoons$ iso citrate

were considered from evidence obtained by the isolation of citric acid as an intermediate. Krebs (1941) had predicted that the fixed carbon would be found in both carboxyl groups of α ketoglutaric acid owing to the symmetrical citrate molecule. In order to account for the fixed carbon only in the carboxyl group α to the carbonyl group Wood et al. (1941b) modified the original Krebs cycle by eliminating citric acid as an intermediate.

The results of these liver tissue experiments point to the confirmation of the Wood and Werkman reaction.

(a) Carbon dioxide was found to be fixed exclusively in the carboxyl groups of the C_4 dicarboxylic acids formed from pyruvic acid. The α and β carbon atoms contained no fixed carbon dioxide.

(b) In the presence of malonate, succinate was shown to contain little or no fixed carbon, whereas the other C_4 dicarboxylic acids did contain fixed carbon. This indicates that there is an aerobic mechanism for the formation of succinate from oxalacetate. Krebs' cycle adequately accounts for the aerobic formation of succinate. In the presence of malonate the anaerobic reduction of oxalacetate to malate is not inhibited. The occurrence of fixed carbon in these two acids is, therefore, accounted

for. None of these investigations, however, is rigid proof that the fixation reaction occurs by carboxylation of pyruvate.

Evans (1942) employed yeast carboxylase and found no exchange of $C^{11}O_2$ with pyruvate during its decarboxylation to acetaldehyde. Ruben and Kamen (1940) have considered this reaction irreversible, however Carson, Ruben, Kamen and Foster (1941) have recently stated that a small amount of $C^{11}O_2$ is utilized with carboxylase preparation in the presence of acetaldehyde and pyruvic acid. Other than these reactions, no information is at hand regarding exchange reactions involving carbon dioxide.

For a complete resumé of the heterotrophic assimilation of carbon dioxide, the reader is referred to the excellent reviews by Werkman (1939) and Werkman and Wood (1942a)(1942b).

METHODS

General Considerations

The search for the enzyme responsible for the fixation of carbon dioxide involves the procurement of a preparation which has other enzymes capable of masking the objective, removed from it. If the fixation occurs by the Wood and Werkman reaction, these interfering enzymes include the

malic dehydrogenase which is responsible for the reduction of oxalacetic acid to malic acid. This reaction, according to Szent-Györgyi (1937) is a reaction of zero order, i.e., the reduction of oxalacetate is very rapid. Another enzyme included in this group is the transaminase of Braunstein and Kritzmann (1937). This enzyme brings about the transfer of an amino group from α amino acids to the keto group of oxalacetic acid and other keto acids. The reaction with oxalacetate is much more rapid than with any other keto acid. Still another enzyme is α keto carboxylase. Since oxalacetate is both an α keto and β keto acid, and the fixation reaction as postulated is only concerned with the β carboxyl group, the α keto carboxylase must be made a limiting factor. The unnamed enzyme which brings about the condensation of pyruvate with oxalacetate, one of the first phases of the Krebs cycle, is another example belonging to this group.

With the hope that the carboxylating enzyme was more stable than the interfering enzymes, a search for a heterotrophic bacterium was made whose enzymatic complexes were stable enough to withstand enzymatic extraction without generally destroying all enzymatic activity. Bacterial physiologists are somewhat at a disadvantage when compared to the mammalian tissue physiologist, in that the latter, by simple mechanical means can disrupt the individual cells

of the tissue and can thereby liberate most of the enzymes. The cell wall, permeable to some metabolites and impermeable to others, has presented a major problem to the bacterial physiologist. In the field of intermediary carbohydrate metabolism many inaccurate conclusions have been drawn. An example will illustrate. The statement is often made that a criterion for a compound to be an intermediate is that it must be attacked as rapidly or more rapidly than the parent compound from which it is derived. It is possible to show with freshly harvested cells of Micrococcus lysodeikticus that phosphopyruvate, a well established intermediate of the Embden-Meyerhof-Parnas scheme of carbohydrate dissimilation, is not attacked. However, by treating the cells so that they become permeable, the phosphate ester is readily attacked.

The exposure of the enzyme system to the environment which the investigator chooses is, however, not the only problem concerned. The cellular metabolism of all cells functions smoothly and appears to carry on a seemingly endless chain of reactions. Under normal conditions there are no breaks in the chain and the accumulation of intermediary products of dissimilation does not occur and their presence is difficult to demonstrate. This appears to be the case especially with aerobic bacteria. By proper manipulation, intermediates can be accumulated in many cases and valuable information obtained.

In addition to possessing a stable enzyme constitution, the bacterium must have potential properties which indicate that it utilizes carbon dioxide. Early work indicated that the enzymatic complexes in Propionibacterium were much too sensitive to withstand treatment which would effect the isolation of the carbon dioxide fixing enzyme.

Fleming (1922) isolated and named Micrococcus lysodeikticus which possessed the ability to become lysed in the presence of the enzyme lysozyme, an enzyme present in both egg albumen and saliva. Penrose and Quastel (1930) reported some of the metabolic properties of the organism and also that lysis had a detrimental effect on most of the dehydrogenases. It was felt that this report suggested the organism might possess the Szent-Györgyi cycle of respiration and also the Krebs cycle for the oxidative removal of pyruvate. To the author both schemes predicate the existence of a mechanism which would supply an abundance of oxalacetic acid. The obvious mechanism was the Wood and Werkman reaction.

For this reason the general metabolic behavior of this organism was investigated, especially as it related to both the Szent-Györgyi scheme and the Krebs cycle.

Specific Considerations

Preparations.

Micrococcus lysodeikticus was grown on a medium containing 1% glucose, 0.5% "Bacto" peptone, 0.3% "Difco" yeast extract, 10% tap water and 2% agar. Inoculations were made on agar slopes in large Roux bottles. After three days' incubation at 30°C., the cells were washed from the agar surface with distilled water, filtered through glass wool and washed twice in a centrifuge.

Untreated cells. The cell paste obtained from centrifugation was made up to a convenient volume so that when transferred to the reaction vessel a final concentration of 2.5% (wet weight basis) was obtained.

Acetone treated cells. Ten grams of cell paste were mixed with 70 ml. ice-cold acetone and shaken thoroughly for five minutes. The cells were filtered off, suspended in 140 ml. ice-cold acetone and shaken vigorously for 10 minutes. The process was repeated. The preparation was then placed in vacuo to remove traces of acetone. These cells possess permeable properties which the untreated cells do not.

Lysed cell preparation. To a 10% cell suspension (wet weight basis) in physiological saline, one-tenth

volume of filtered saliva was added, and the mixture incubated one hour at 36°C. whereupon lysis occurred. For the control a 10% cell suspension without saliva was incubated in like manner.

Diphosphothiamine and Mg^{++}-deficient acetone treated cells. It is known that diphosphothiamine and Mg^{++} are required as coenzymes for the pyruvic acid carboxylase in yeast. The complete enzyme decarboxylates pyruvate to acetaldehyde and carbon dioxide. Likewise, these coenzymes are required for the oxidative decarboxylation of pyruvate to acetic acid and carbon dioxide by mammalian tissue and bacteria. In order to determine whether either or both of these coenzymes are required for the β keto acid carboxylating enzyme, an enzyme preparation deficient in these constituents was prepared. Lipmann (1939) removed the diphosphothiamine from Lactobacillus delbruechii by washing with a phosphate buffer solution at pH 8.0. An adaptation of his method was found successful for removing both the diphosphothiamine and Mg^{++}. One gram of the acetone treated preparation was vigorously washed twice for 10 minutes with 50 ml. of 0.1 M dipotassium hydrogen phosphate and once with 50 ml. of distilled water. This preparation maintains its deficiency when dried on a porous plate, however if stored as a suspension, partial recovery occurs which may result from residual amounts of diphosphothiamine and Mg^{++} reorienting

on the carboxylase protein, or to the resynthesis of diphosphothiamine from its component parts. The two coenzymes cannot be removed by the phosphate washing unless the cells are first treated with acetone.

Diphosphothiamine, Mg^{++} and phosphate deficient acetone treated preparation. To determine the phosphate requirements of the carboxylating enzyme, a phosphate deficient preparation was first prepared. One gram of the acetone preparation was washed twice with 50 ml. of 0.05 M borate at pH 8.5, at 10 minute intervals. The washing was followed by three additional washings with 50 ml. 0.1 M acetate buffer pH 4.5, also at 10 minute intervals, and then a 10 minute washing with distilled water. The shaking was done mechanically. This treatment also removes the diphosphothiamine and Mg^{++}.

Diphosphothiamine and Mg^{++}-deficient preparation of lysed cells. To 4 ml. of a 20% solution of lysed cells, 6 ml. of a saturated solution of $(NH_4)_2SO_4$ were added. The mixture was vigorously shaken by hand for 10 minutes and centrifuged. The precipitate was made up to a volume of 4 ml. with distilled water and the $(NH_4)_2SO_4$ washing repeated twice. Finally the precipitate was washed with distilled water and made up to the original volume of 4 ml.

Cytochrome c deficient preparation. Ten grams of cell

paste were sufficiently diluted with physiological saline and one-tenth volume filtered saliva so that the final concantration was 20% (wet weight basis). The suspension was incubated one hour at 36°C. in order to bring about lysis. Thirty ml. 0.04 M phosphate buffer pH 7.0 was added and shaken mechanically for 1.5 hours. The suspension was then centrifuged slowly to remove the macroscopic particles. The cloudy liquid was then treated with 30 ml. 0.2 M acetate buffer pH 4.5, centrifuged thoroughly. The clear liquid was discarded and the solids resuspended in sufficient 0.1 M phosphate buffer, pH 7.3 so that when they were transferred to the reaction vessel, a final concentration of 2.5% was obtained. The cytochrome c concentration of this suspension is much smaller than that of the untreated lysed cell preparation as determined spectroscopically.

Beef heart cytochrome c. The method of Keilin and Hartree (1937) was used.

Preparation of oxalacetic acid. The following method for the preparation of oxalacetic acid has been found to be much less laborious than the methods given in the literature.

To the sodium salt of diethyloxalacetate (100 gm. in 250 ml. water) concentrated H_2SO_4, equivalent to the sodium, is added. The free ester is removed as an oily layer by means of a separatory funnel and dissolved in 80 ml. ether. The ether solution is washed twice with 50 ml. distilled

water, and the ether evaporated off. The ester is hydrolyzed by treatment with three volumes of concentrated HCl at room temperature. The acid solution is vigorously shaken mechanically for two hours. The solution is then placed at -26°C. for 24 to 48 hours. The precipitated oxalacetic acid is removed by filtration, dissolved in a requisite amount of warm acetone and recrystallized by cooling and the addition of chloroform. The HCl solution still contains quantities of the diethyloxalacetate. One additional volume of concentrated HCl is added and the shaking and cold treatment repeated. The purity of the oxalacetate as determined by the aniline-citrate method (Edson, 1935) is greater than 98%.

Heavy carbon sodium bicarbonate. $NaHC^{13}O_3$ was prepared from $C^{13}O_2$ obtained from methane whose C^{13} had been concentrated in a thermal diffusion column according to Nier and Bardeen (1941).

Analytical methods.

The Barcroft-Warburg respirometer was used in this study. All reactions were carried out at 30.4°C. The conventional 18 ml. flask possessing two side-arms was used in all experiments with the exception of those in which exchange experiments were conducted. In the latter 125 ml., two side-arm, flasks were employed. The volumes of the

reactant mixtures will be designated in the appropriate tables and figures.

Phosphate buffers were made according to Clark (1928). The reactants of the exchange reactions were buffered with a mixture of 0.021 phosphate, pH 6.6 and 0.05 M $NaHC^{13}O_3$ in a total volume of 30 ml. The approximate pH was 7.2. Appropriate concentrations of the two buffers were placed in the side arms of the flask, and mixed with the enzyme preparation and substrate after temperature equilibrium had been obtained. Three hundred mg. of the acetone preparations were used in each flask. The concentration of the oxalacetate was 0.053 M and of all other acids, 0.026 M. Each was adjusted to pH 7.0 before addition. Owing to the instability of oxalacetate at acid reactions and the length of time required for analyses, the concentration of this acid was doubled so that appreciable quantities remained for analysis. At the conclusion of the reaction time, the enzyme preparation was rapidly centrifuged and analyses made on the supernatant liquid. In all experiments except when ether extractions were first required, the supernatant liquid was transferred to a 20 cm. x 5 cm. pyrex tube fitted with a sintered glass disc for aeration. Arrangements were made for the addition of the necessary reagents during the course of the aeration.

Pyruvic acid determination. Pyruvic acid was determined

by the carboxylase method (Krebs et al., 1940) and also by the colorimetric salicylaldehyde method of Straub (1936). The carboxyl group of pyruvic acid was obtained by ceric sulphate oxidation (Fromageot and Desnuelle, 1935). The products of this oxidation are acetic acid and carbon dioxide, the latter originating from the carboxyl group of the pyruvic acid.

Removal of the residual $NaHC^{13}O_3$. The instability of oxalacetic acid to acidification and heat complicates the usually simple procedure of removing the residual $NaHC^{13}O_3$. The removal of this $NaHC^{13}O_3$ is important since traces of the material will interfere with subsequent isotope determination made on the carboxyl carbons of the various substrates. Preliminary experiments conducted with the phosphate-bicarbonate buffer mixture and oxalacetate gave quantitative recoveries of the bicarbonate upon acidification to congo red, and aeration through a sintered glass disc for 15 minutes at room temperature. In order to insure that traces of the $NaHC^{13}O_3$ did not remain, 0.75 mM $NaHC^{12}O_3$, i.e., bicarbonate with the normal complement of C^{13} was added and the aeration procedure repeated. The C^{13} content of this normal bicarbonate "rinse" was determined and compared with the C^{13} concentration of the acid analyzed.

Oxalacetic acid determination. To determine whether exchange in oxalacetic acid occurs in the carboxyl group adjacent to the methylene group, or in that adjacent to the

carbonyl group, the acid was decarboxylated by two methods.

(1) Aniline-citrate method (Edson, 1935).

The reaction mixture was made acid with a 50% citric acid solution. One volume of the acid to seven volumes of the reaction mixture is sufficient. The residual CO_2 was removed by aeration for 15 minutes at room temperature. The $NaHC^{12}O_3$ "rinse" was next added and the aeration procedure repeated. One volume of citrate-aniline mixture (1:1) was added to every five volumes of the original reactant mixture, and the CO_2 derived was collected in 1.5 N carbonate-free NaOH. This CO_2 originates from the carboxyl group adjacent to the methylene group. The other carboxyl carbon is linked to the resulting pyruvanilide, consequently it is inadvisable to analyze for it. Acetoacetic acid interfers with the oxalacetic acid in this determination, however if the reaction is carried out at 5°C. only the oxalacetic acid will be determined. No acetoacetic acid was formed under the conditions of the experiments.

(2) Acid-heat decarboxylation.

When oxalacetate is made acid to congo red with H_2SO_4 and boiled for 30 minutes, it is quantitatively decarboxylated to pyruvic acid and carbon dioxide. This CO_2 originates from the carboxyl group adjacent to the methylene group. The advantage of this method is that the resulting pyruvate can be oxidized with ceric sulphate, thereby obtaining the carbon

associated with the carboxyl group adjacent to the carbonyl group.

α ketoglutaric acid determination. α ketoglutaric acid was oxidized with $KMnO_4$. (Wood et al. 1941b). The products of this oxidation are succinic acid and carbon dioxide, the latter originates from the carboxyl group adjacent to the carbonyl group.

Lactic acid and pyruvic acid separation and their determinations. Lactic and pyruvic acids were separated by continuous ether extraction in the presence of sodium bisulphite. The ether extract contains the lactic acid. The pyruvate-bisulphite complex was decomposed by boiling, and the extraction repeated to recover the pyruvic acid. The carboxyl group of the lactic acid was obtained by $KMnO_4$ oxidation according to Friedemann and Graeser (1933) and that of the pyruvate by ceric sulphate oxidation.

C^{13} determinations. C^{13} concentrations were determined on the mass spectrometer (Mier, 1940). All materials used for mass spectrometric analyses were converted to carbon dioxide collected in 1.5 N carbonate-free NaOH.

Q_{O_2} values represent the number of microliters of oxygen consumed by one milligram of cellular material (dry weight basis) in one hour. Calculations of these values were made by the following equations:

$$Q_{O_2} = \frac{\mu l.\ O_2}{\text{mg. cells (dry weight basis) x hours}}$$

Respiratory quotient is defined as the ratio of microliters of carbon dioxide evolved to the microliters of oxygen consumed during the same interval of time.

EXPERIMENTAL

Respiratory Properties of *Micrococcus lysodeikticus*

Aerobic and anaerobic dissimilation by untreated cells.

Micrococcus lysodeikticus is a facultative anaerobic organism which grows slowly under conditions of reduced oxygen tension but grows luxuriantly under highly aerobic conditions. It requires complex nitrogenous and carbonaceous materials for growth. The medium prepared, as stated in the section on methods, was found very satisfactory for obtaining the non-proliferating cells used in these experiments.

The aerobic and anaerobic behavior of the freshly harvested cells on glucose, pyruvate and the C_4 dicarboxylic acids is shown in Table 1.

The oxygen uptake on glucose is somewhat smaller than on pyruvate which is a key intermediate in the Embden-Meyerhof-Parnas scheme of carbohydrate dissimilation. On the C_4 dicarboxylic acids, intermediates of the Krebs cycle

Table 1.

Anaerobic and Aerobic Metabolism of Untreated Cells of *M. lysodeikticus* on Glucose, Pyruvate and the C_4 Dicarboxylic Acids

Substrate 0.025 M	Aerobic					Anaerobic
	µl. O_2 uptake	µl. CO_2 evolution	Experimental R.Q.	Theoretical R.Q. for complete oxidation	Q_{O_2}	µl. CO_2 evolution
Glucose	128	132	1.03	1.0	6.4	7
Pyruvate	248	305	1.23	1.2	12.4	6
*Oxalacetate	225	279	1.24	1.6	11.2	4
Malate	250	340	1.36	1.33	12.5	2
Fumarate	242	331	1.37	1.33	12.1	3
Succinate	264	302	1.14	1.14	13.2	1

Total volume of reactants = 2 ml.
2.5% cell suspension (wet weight basis). 0.125 PO_4 buffer pH 6.8.
Aerobic experiments contained air in gas space, anaerobic contained N_2.
Temperature 30.4°. Time, one hour.
*All values corrected for spontaneous decarboxylation of oxalacetate.

and catalysts in the Szent-Györgyi scheme, the Q_{O_2} values are high as compared to glucose. The respiratory quotient of glucose is one which indicates that the oxidation of glucose is complete to carbon dioxide and water. Likewise, the respiratory quotients of the other substrates are such that complete oxidation is indicated. The high Q_{O_2} values for the C_4 acids suggest that they may be functioning in the respiratory processes of the organism.

Szent-Györgyi scheme and carbon dioxide fixation.

Of interest are the anaerobic experiments illustrated in Table 1, in that there is no evolution of carbon dioxide. This is especially true in the case of malate and fumarate. If the Szent-Györgyi scheme was functioning, i.e., these metabolites acting as coenzymes for hydrogen transport, one would expect these substrates to be metabolized anaerobically. Penrose and Quastel (1930) have shown that M. lysodeikticus contains fumarase, the enzyme capable of promoting the following equilibrium whose constant is approximately 3.0.

$$\underset{\text{fumaric acid}}{COOH.CH{:}CH.COOH} \underset{-HOH}{\overset{+HOH}{\rightleftharpoons}} \underset{\text{malic acid}}{COOH.CH_2.CHOH.COOH}$$

With either acid acting as the substrate the malate should be oxidized at the expense of the fumarate. The reactions are:

$$\underset{\text{malic acid}}{COOH.CH_2.CHOH.COOH} + \underset{\text{fumaric acid}}{COOH.CH{:}CH.COOH} \longrightarrow \underset{\text{oxalacetic acid}}{COOH.CO.CH_2.COOH} + \underset{\text{succinic acid}}{COOH.CH_2.CH_2.COOH}$$

The oxalacetic acid should be decarboxylated, rapidly if the decarboxylation is enzymatic or slowly if spontaneous, to pyruvate and carbon dioxide. However, no carbon dioxide was formed. Analyses made for the presence of oxalacetate and succinate were also found to be negative.

Ogston and Green (1935) have shown that oxalacetic acid has an inhibitory effect on the succinic and malic acid dehydrogenases. To avoid this possible inhibition the same experiment was performed in the presence of semicarbazide, a keto fixative. However, no oxalacetate was found under these conditions. It is evident from these experiments that these anaerobic oxidations do not occur in M. lysodeikticus.

Krebs and Eggleston (1940b) have expressed some doubt as to the fumarate $\xrightarrow{+2H}$ succinate step. They prefer the theory whereby all succinate is derived by oxidative reactions including α ketoglutaric acid. Other investigators have not favored the oxalacetate $\xrightarrow{+2H}$ malate step, especially when the hydrogens involved originate from the oxidation of triose. The triose dehydrogenase is a diphosphopyridine nucleotide dependent enzyme, as is the malic dehydrogenase. The Szent-Györgyi scheme explains the progressive transfer

of hydrogen toward oxygen and since dihydrodiphosphopyridine nucleotide is the reduction product of both the oxidation of triose and malic acid, there appears no necessity of the oxalacetate $\underset{-2H}{\overset{+2H}{\rightleftharpoons}}$ malate step. However, Wood et al. (1942) have shown that in malonate poisoned liver tissue, pyruvate is oxidized and that fumaric and malic acids may be isolated with fixed carbon in the carboxyl groups. Obviously these acids are the anaerobic reduction products of oxalacetate and under the proper redox conditions, the reductions take place.

By employing techniques with M. lysodeikticus similar to those used by the Szent-Györgyi school on muscle tissue preparations, the demonstration of C_4 acid catalysis has been partially demonstrated. This technique involves the preparation of tissue whose C_4 acid content has been made the limiting factor, i.e., the concentration of these acids is so low that hydrogen transport via these acids is greatly impaired. The addition of these acids to such a preparation, in the presence of glucose as the substrate, greatly enhances the oxidation of the glucose.

Results of such an experiment are given in Table 2 with freshly harvested cells of M. lysodeikticus which were washed three times with distilled water to remove residual C_4 dicarboxylic acids. Results obtained on the addition of small concentrations of fumarate to the glucose oxidation

Table 2.

C_4 Dicarboxylic Acid Catalysis of the Oxidation of Glucose

by Untreated Cells of *M. lysodeikticus*

Time interval	0.025 M glucose μl. O_2 uptake	0.005 M fumarate μl. O_2 uptake	0.025 M glucose + 0.005 M fumarate μl. O_2 uptake	Stimulation %	Type of cell
15 minutes	23	14	51	38	Washed 3 times
	11	6	22	30	Washed 72 hours
30 minutes	61	32	134	45	Washed 3 times
	33	14	66	40	Washed 72 hours
60 minutes	142	60	204	0	Washed 3 times
	77	36	114	0	Washed 72 hours

Total volume of reactants = 2 ml. 2.5% cell suspension (wet weight basis). 0.125 M PO_4 buffer pH 7.2; air in gas space. Temperature 30.4°, Time, one hour

suggests that the C_4 acids are acting catalytically since this oxygen uptake is greater than the sum of the glucose and fumarate oxidations during the first 30 minutes. If these results are compared to those obtained by Gözsy and Szent-Györgyi (1934) with pigeon breast muscle in which stimulations of high order, 100 to 600%, were attained they do not adequately support the theory that the C_4 acids act as hydrogen transport catalysts. Banga (1937) has also shown that the C_4 acids are tenaciously held to the protein moiety of the enzyme, and that only after long periods of dialysis is the catalytic effect shown. Accordingly, several experiments were conducted wherein the freshly harvested cells were exhaustively washed with distilled water for periods as long as 72 hours. Table 2 also gives the results of these experiments. With the exception that the general enzymatic activity is slightly lower, these results are comparable to the former experiments. These results indicate that the cells contain a mechanism for replenishing the C_4 acid content. This proposal would be suggested by the small stimulations over a short period of time, i.e., the synthesis of oxalacetate by the Wood and Werkman reaction would restore the C_4 acid content to the original value, thereby destroying the catalytic effect of the added C_4 acids. It has already been mentioned that muscle tissue does not have the property of utilizing carbon dioxide, consequently the

stimulations obtained with that tissue would be of a higher order and for a longer period of time. It will be noticed in Table 2 that the oxidation of glucose by M. lysodeikticus is not linear; the rate of oxidation increases which suggests that the C_4 acids are being synthesized, thereby bringing about a restoration of respiration to a higher level. Whether or not the C_4 acids are respiratory catalysts in M. lysodeikticus as Szent-Györgyi assumes in muscle tissue can not be answered now, however, from the evidence presented here it is obvious that they have a respiratory function.

Banga et al. (1939) have reported the need of C_4 dicarboxylic acids for the oxidation of pyruvic acid with pigeon brain dispersions. The oxidation product was not defined; however, with the addition of adenylic acid the oxidation proceeded beyond the acetic acid stage as judged by the respiratory quotient. The catalytic effect was only for a short period of time, 20 minutes, and then began to fall abruptly. According to these authors this result was due to the general destruction of all enzymatic activity, especially the pyruvate dehydrogenase.

In order to obtain a preparation comparable to the muscle brei and the brain dispersions whose C_4 acid content could be lowered by dialysis, freshly harvested cells were treated with acetone. Previous experiments had shown these cells to possess a highly permeable cell wall.

Table 3 shows the activity of this preparation on glucose, pyruvate and the C_4 dicarboxylic acids. With the exception of glucose and succinate the oxygen uptake is similar to the untreated cells. The pyruvate activity is somewhat lower than the untreated cell owing to cessation of oxidation at the acetic acid stage and is not complete as in the untreated cells. Anaerobically and aerobically the action of this preparation on oxalacetate is significant and will be discussed later in detail.

Attempts to show the catalytic effect of C_4 acids with this preparation could only be made with pyruvate oxidation, since glucose was not oxidized by the preparation. The diphosphothiamine and Mg^{++} deficient preparation, mentioned in the methods section, was assumed to be deficient in the C_4 acids. This assumption was based on the premise that diphosphothiamine, which is a much larger molecule than any of the C_4 acids, was removed by the alkaline phosphate washing. The oxidation of pyruvate, after addition of Mg^{++} and diphosphothiamine to the deficient preparation, was restored to the value obtained with the unwashed preparation (Table 4). The oxygen uptake with 0.005 M fumarate is also given. In the presence of pyruvate, diphosphothiamine, Mg^{++} and 0.005 M fumarate, the oxygen uptake indicates simply an addition effect but no catalytic effect. If the latter occurred, the oxygen uptake would be significantly greater

Table 3.

Anaerobic and Aerobic Metabolism of Acetone Treated Cells of *M. lysodeikticus*

Substrate 0.025 M	Aerobic		Anaerobic
	µl. O_2 uptake	µl. CO_2 evolution	µl. CO_2 evolution
Glucose	8	6	0
Pyruvate	178	320	12
*Oxalacetate	162	1220	915
Malate	248	312	3
Fumarate	235	294	2
Succinate	12	0	0

Total volume of reactants = 2 ml. 20 mg. acetone treated cells per cup. 0.125 M PO_4 buffer pH 6.8 Aerobic experiments contained air in gas space, anaerobic contained N_2. Temperature 30.4°C. Time, one hour.
*Corrected for spontaneous decarboxylation of oxalacetate.

Table 4.

Effect of C_4 Acid Catalysis on the Oxidation of Pyruvate to Acetate

by a C_4 Acid Deficient Acetone Preparation of *M. lysodeikticus*

Unwashed preparation	Deficient preparation			
0.025 M pyruvate: $\mu l.$ O_2 uptake	0.025 M pyruvate: $\mu l.$ O_2 uptake	0.005 M fumarate: $\mu l.$ O_2 uptake	0.005 M fumarate + 0.023 M pyruvate: $\mu l.$ O_2 uptake	Stimulation %
172	169	68	242	2

Total volume of reactants = 2 ml. 20 mg. acetone preparation per cup.
25 µg. diphosphothiamine. 0.006 M $MgCl_2$. 0.125 M PO_4 buffer pH 6.8.
Air in gas space.
Temperature 30.4°C. Time, one hour.

than the sum of the results with pyruvate and fumarate. The conclusion reached is that the C_4 acids are not acting as hydrogen transporters during the pyruvate to acetate oxidation in M. lysodeikticus. It is quite possible in the experiments performed by Banga et al. (1939) with brain dispersions, that the C_4 acids were catalyzing the oxidation of pyruvate beyond the acetic acid stage. If the removal of acetic acid involves a cycle analagous to the Krebs cycle the action of the C_4 acids is readily understandable.

Krebs' cycle and carbon dioxide fixation.

The possibility of the C_4 dicarboxylic acids acting as respiratory intermediates in a cycle of carbohydrate dissimilation is a much more attractive hypothesis. The Krebs cycle which has been previously described is an example. Repeated experiments, devised in such manner as to facilitate the accumulation of the intermediates of this cycle in quantities large enough to determine quantitatively have failed with M. lysodeikticus. However, positive qualitative tests for succinic, citric and possibly a ketoglutaric acids have been obtained. Evidently the equilibria in the metabolism of an aerobic organism of this type, are such that the reduced substrate is always in excess and that accumulation of the oxidized product greatly inhibits the further oxidation of the reduced substrate. Kalckar (1941)

has given an example of this type with a possible explanation. As a rule, the hydrogen acceptor (oxidant) of an oxidoreduction system must have a more positive value than the hydrogen donator (reductant). Hydrogen transfer systems which transfer hydrogen from the hydrogen donator to the acceptor usually have redox potentials between those of the hydrogen donator and of the hydrogen acceptor. Exceptions, however, are known. The normal potential of the transfer system triphosphopyridine nucleotide is considerably lower than that of the system glutamic acid $\underset{+2e}{\overset{-2e}{\rightleftharpoons}}$ α ketoglutaric acid; nevertheless, this transfer system can accept the hydrogen of glutamic acid and transfer it to a more positive system. The reason is that in the presence of oxygen practically all of the pyridine nucleotide is kept in the oxidized form, which has a redox potential at the proper level, to accept hydrogen (electron). In other words the oxidant and reductant are not being compared at the same percentage of oxidation. In the absence of oxygen the dehydrogenation of glutamic acid by the nucleotide stops immediately.

In view of the observation that the acetone treated cells oxidize pyruvate only to acetic acid and that the C_4 acids have no catalytic effect on this oxidation, the suggestion is made that between pyruvate and acetate there exists an intermediate which by further reactions can be oxidized to completion by the untreated cell. The hypothesis presented

here is that this intermediate, probably acetyl phosphate (Lipmann, 1941), condenses with oxalacetate to form one of the six carbon compounds of the Krebs cycle. The oxalacetate is the result of the carboxylation of pyruvic acid. In the acetone preparation the enzyme bringing about this condensation to form the six carbon compound is destroyed. The C_4 dicarboxylic acid catalysis obtained during the oxidation of glucose by the untreated cells could adequately be explained by the Krebs cycle. The regeneration of oxalacetate is paramount for its operation and with the addition of the C_4 acids this is accomplished through their oxidation to oxalacetate. The carboxylation of pyruvate by the Wood and Werkman reaction is an alternate method of supplying oxalacetate, consequently the reaction is an important link in respiratory processes. The fact that the evidence for the presence of the Krebs cycle in bacteria is meager, does not minimize the necessity for an adequate supply of the C_4 acids for respiratory processes.

Properties of the cytochrome system of Micrococcus lysodeikticus.

The cytochrome system is a necessary adjunct to the respiratory system of most aerobic forms of life. It represents the most terminal portion of the chain of catalysts concerned in respiration. According to Keilin (1925) it

consists of three cytochrome components a, b and c and cytochrome oxidase. The latter activates molecular oxygen so that the reduced component c becomes oxidizable. Very little is known about the components a and b, however the c component has been isolated in pure form (Keilin and Hartree, 1937). This component is readily oxidized and reduced, the oxidation is brought about by cytochrome oxidase and the reduction by some of the reducing enzymes in the cell. Since the cytochrome system is a major portion of the respiratory processes, this system has been studied in M. lysodeikticus. It is generally thought that cytochrome oxidase is sensitive to acetone treatment. According to Szent-Györgyi (1937) only succinic acid is capable of donating hydrogen to cytochrome c, consequently the failure of the acetone preparation to oxidize succinate could be due to the destruction of cytochrome oxidase by the acetone. However, oxidations carried out by the preparation are sensitive to cyanide (Table 5), and this result suggests that the iron containing catalyst, cytochrome oxidase, is intact and also that the oxidations do not take place via the fumarate-succinate system.

Quastel and Wheatley (1938) have shown that sodium ferricyanide acts as an hydrogen acceptor for the succinate system under anaerobic conditions. The reduction of ferricyanide forms one hydrogen ion equivalent according to the equations:

Table 5.

Cyanide Sensitivity of Oxidations Performed by Acetone Preparation of *M. lysodeikticus*, and Effect of Acetone Treatment on Dehydrogenases as Tested by Ferricyanide Technique

Substrate 0.025 M	µl. O_2 uptake			µl. CO_2 evolved
	Normal oxidation	0.002 M KCN	Inhibition %	
Pyruvate	178	8	95.5	901
Oxalacetate	162	7	95.1	864
Malate	248	10	95.9	672
Fumarate	235	7	97.0	638
Succinate	12	2	-	46

Total volume of reactants = 2 ml. 20 mg. cells per cup. 0.125 M PO_4 buffer pH 6.8. Air in gas space. Ferricyanide experiments contained 0.025 M $NaHCO_3$ buffer and 95% N_2 and 5% CO_2 in gas space.
CO_2 was absorbed by Krebs' mixture in cyanide experiments (Krebs, 1935).
Temperature 30.4°C. Time, one hour.

$H + Fe(CN)_6^{---} \longrightarrow H^+ + Fe(CN)_6^{----}$

$H^+ + HCO_3^- \longrightarrow H_2CO_3 \longrightarrow CO_2 + H_2O$

The hydrogen ion in the presence of bicarbonate liberates carbon dioxide which may be measured manometrically. The results (Table 5) show that the succinate dehydrogenase is almost entirely destroyed by acetone treatment when examined by the ferricyanide technique.

Spectrographic observations of the acetone preparation show the typical reduced cytochrome component absorption spectrum. The components a, b and c are present in their typical positions, 5500 Å, 5660 Å and 6040 Å respectively. The substrates listed in Table 3 rapidly reduce oxidized cytochrome c with the exception of glucose and succinate. The reduced component is rapidly oxidized when placed under aerobic conditions which indicates the presence of cytochrome oxidase. The failure of glucose or succinate to reduce oxidized cytochrome c shows the destruction of the succinate dehydrogenase, and also possibly the triose dehydrogenase. Of interest in this connection is that both the triose dehydrogenase and the succinate dehydrogenase are SH proteins and the acetone may have an injurious effect on this type of protein.

The presence of cytochrome oxidase is further demonstrated in Table 6. p-Phenylenediamine reduces oxidized cytochrome c non-enzymically. If cytochrome oxidase is

Table 6.

p-Phenylenediamine and Hydroquinone Oxidation by an Acetone Preparation of *M. lysodeikticus*

Substrate 14 mg./cup	µl. O_2 uptake		
	Normal oxidation	0.002 M KCN	Inhibition %
p-Phenylenediamine	352	22	94
Hydroquinone	10	8	-

Total volume of reactants = 2 ml. 20 mg. cells per cup. 0.125 PO_4 buffer pH 7.3. Air in gas space. Temperature 30.4°C. Time, one hour.
CO_2 was absorbed by Krebs' mixture in cyanide experiments (Krebs, 1935).
Values are corrected for autoxidation of substrates.

present the reduced chtyochrome c is oxidized and the cycle is repeated; this results in an oxygen uptake. The oxygen uptake in the presence of the diamine was 352 µl./hour and the cyanide inhibition was almost 100%.

Stotz et al. (1938) have found that hydroquinone behaves similarly to p-phenylenediamine, in mammalian tissue preparations and that owing to its more favorable redox potential, + 280 mv., hydroquinone provides a more specific test for cytochrome oxidase from mammalian tissue. The redox potential of cytochrome c is + 270 mv. (Ball, 1938) and that of p-phenylenediamine is less than -40 mv. The somewhat autoxidizable b component of cytochrome, according to Ball (1938), has a potential more negative than hydroquinone but more positive than p-phenylenediamine. Consequently oxidation of hydroquinone does not proceed through the autoxidizable b component. The fact that p-phenylenediamine oxidation, in the experiments given in Table 6, was 94% inhibited by KCN is proof that the oxidation does not significantly proceed through the autoxidizable cytochrome b. Hydroquinone is not oxidized and neither does it reduce cytochrome c of M. lysodeikticus when viewed spectroscopically, whereas p-phenylenediamine does. An explanation of these results can be made on the basis of redox potentials. The tetrapyrrolic portion of the c component when

attached to a chemically defined nitrogen base as nicotine, exhibits the same α band at 5500 Å as when attached to its natural protein moiety. The soret bands, however differ as would be expected. The same situation may exist in M. lysodeikticus, namely that the protein moiety of the cytochrome c component differs from that obtained from yeast and mammalian tissue. The soret band of this bacterial cytochrome has never been observed. The suggestion is therefore made that the protein moiety of the bacterial cytochrome c alters the redox potential of the component so that it is more positive than p-phenylenediamine, however more negative than hydroquinone.

Further evidence that the cytochrome c component of M. lysodeikticus and that of heart muscle are dissimilar can be obtained from the lysed preparation which is deficient in cytochrome c. Keilin and Hartree (1938) have obtained cytochrome oxidase preparations sufficiently devoid of the soluble cytochrome c component that oxygen uptake on p-phenylenediamine was almost nil. With addition of cytochrome c, isolated from beef heart, oxygen uptake ensued. The oxygen uptake of the cytochrome c deficient bacterial preparation on p-phenylenediamine is given in Table 7. The activity has been much decreased as compared to the acetone preparation quoted in Table 6. The addition of beef heart cytochrome c to the deficient preparation does not increase

Table 7.

Oxidation of p-Phenylenediamine and Hydroquinone in the Presence of a Bacterial Cytochrome c Deficient Preparation

Inhibitor	Hydroquinone	p-Phenylene-diamine	Beef heart cytochrome c +	
			p-Phenylenediamine	Hydroquinone
	µl. O_2 uptake	µl. O_2 uptake	µl. O_2 uptake	µl. O_2 uptake
None	10	64	68	12
KCN 0.002 M	8	12	14	9

Total volume of reactants = 2.0 ml. 0.125 M PO_4 buffer pH 7.3. 20 mg. cell preparation per cup. 14 mg. p-phenylenediamine or hydroquinone per cup. 0.125% beef heart cytochrome c.
Temperature 30.4°C. Time, one hour.

the oxidation of the diamine which indicates that cytochrome c from beef heart will not replace the bacterial cytochrome c. The latter also requires a specific cytochrome oxidase.

It is felt that the results of these respiratory studies, which pertained to the C_4 dicarboxylic acid catalysis and also the cytochrome system in M. lysodeikticus justified the assumption that this organism possesses respiratory properties very similar to the mammalian tissue. It has already been noted that the Wood and Werkman reaction has an important role in liver tissue respiration.

Demonstration of Oxalacetate Carboxylase

Permeability as an obstacle to demonstration.

The behavior of the acetone preparation on oxalacetate merits special attention in view of the possibility of carbon dioxide fixation (cf. Table 3). Anaerobically oxalacetate is decarboxylated very rapidly in the presence of this preparation. Since there was no increase in carbon dioxide evolution anaerobically in the presence of the untreated cells and oxalacetate (Table 1), it may be assumed that the oxalacetate did not reach the active enzyme centers. Apparently the acetone extraction of the cells makes the cell membrane permeable to oxalacetate. Objections to this explanation may be made inasmuch as the untreated cells showed an oxygen

uptake with oxalacetate (Table 1). It is probable, however, that the oxalacetate did not penetrate the untreated cell even in the aerobic experiments, and that the oxygen uptake was due to the oxidation of pyruvate formed by the spontaneous decarboxylation of oxalacetate. This explanation is supported by the fact that anaerobically, with the acetone treated cells, the quantity of carbon dioxide from oxalacetate (915 µl.) is substantially equivalent to the carbon dioxide from the corresponding aerobic experiment (1220 µl.) minus the carbon dioxide from the aerobic pyruvate experiment (320 µl.). Apparently the excess carbon dioxide in the aerobic experiment is the result of pyruvate dissimilation. Pyruvate is not dissimilated anaerobically, consequently the carbon dioxide evolved from oxalacetate under these conditions must have arisen by its decarboxylation, shown in Table 3 by the 915 µl. of carbon dioxide obtained anaerobically from 928 µl. of oxalacetate.

Objections may also be raised that if the other C_4 acids reach their enzyme centers, oxalacetate should likewise do so. Apparently other compounds having the grouping $-CO.CH_2.CO-$ do not penetrate the cell wall, e.g., malonic acid, generally recognized as an inhibitor of succinate oxidation, shows no inhibition with M. lysodeikticus, even after long incubation before succinate addition. Apparently, the malonate does not penetrate the cell to the enzyme center.

This hypothesis cannot be tested with the acetone preparation inasmuch as it does not oxidize succinate.

Results given in Figure 1 further substantiate the cell permeability hypothesis. The decarboxylation of oxalacetate by untreated cells, by previously lysed cells and by cells that were lysed during the course of the experiment is compared. If cell permeability is limiting the decarboxylation of oxalacetate, it is to be expected that as lysis proceeds the enzyme will become liberated and be available for decarboxylation. Decarboxylation by the untreated cell was identical with the spontaneous decarboxylation; therefore the two curves are superimposable. The lysed preparation, however showed a remarkable ability to decarboxylate oxalacetate, and the experiment in which lysis proceeded in the presence of oxalacetate showed an increasing rate of decarboxylation. Saliva, the lysing agent, alone does not increase the spontaneous decarboxylation. The results of these experiments clearly establish the importance of cell permeability.

Products of the decarboxylation of oxalacetate.

Pyruvic acid and carbon dioxide are the products formed by the anaerobic breakdown of oxalacetate (Table 8). For each mole of oxalacetic acid decarboxylated one mole of pyruvate and one of carbon dioxide were formed. The pyruvate

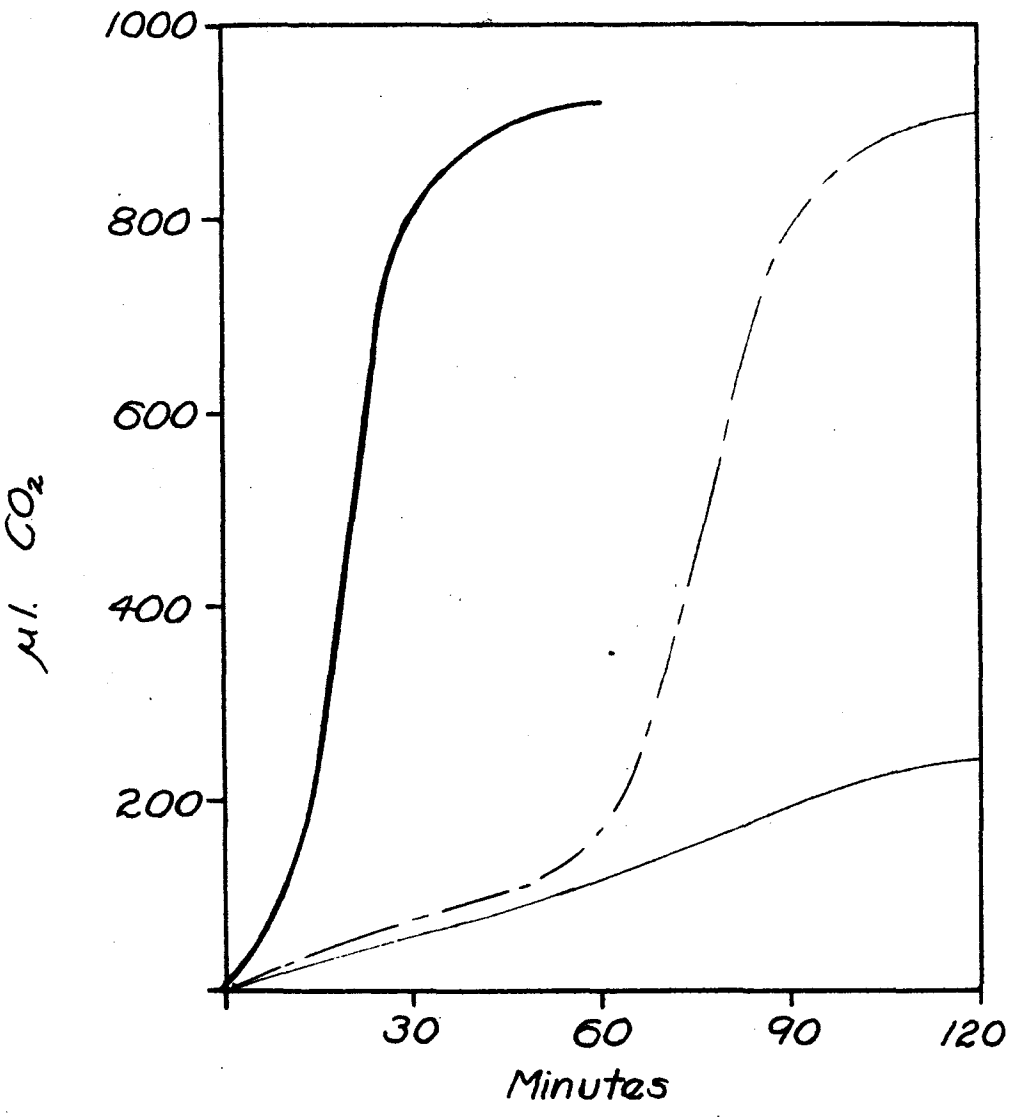


Fig. 1 Effect of Lysis on the Decarboxylation of Oxalacetate.

——— 2.5 % lysed cells + q20 µl. oxalacetate.
2.5 % untreated cells + 0.1 volume saliva
— - — + q20 µl. oxalacetate.
——— 2.5 % untreated cells + q20 µl. oxalacetate.

Table 8.

Products of Anaerobic Decarboxylation of Oxalacetate

by an Acetone Preparation

Material determined	Method of determination	Quantity obtained (µl.)	Recovery %
Oxalacetate dissimilated	Aniline citrate	998	-
Pyruvate	Carboxylase	958	97
Pyruvate	Salicylaldehyde	970	98
CO_2	Acidification	947	96

determinations by the carboxylase and the salicylaldehyde methods agreed, and there was an equivalent recovery of carbon dioxide on acidification. The oxalacetate was determined by the aniline-citrate method.

Effects of Mg^{++} and diphosphothiamine on bacterial carboxylase of oxalacetic acid.

The question next arises as to the relationship between oxalacetate carboxylase and pyruvate carboxylase. Krebs and Eggleston (1940b) and Smyth (1940) are of the opinion that the two are identical or at least similar. It is well known that yeast carboxylase not only decarboxylates pyruvate but also oxalacetate. The products of the decarboxylation of the latter are acetaldehyde and two molecules of CO_2. There are two possible mechanisms by which yeast carboxylase may decarboxylate oxalacetate: (1) initial decarboxylation of the carboxyl group adjacent to the methylene group or (2) initial decarboxylation of the carboxyl group adjacent to the carbonyl group. These reactions are illustrated below:

(1) $\underline{COOH}.CH_2.CO.COOH \longrightarrow CH_3.CO.COOH + CO_2$

$CH_3.CO.\underline{COOH} \longrightarrow CH_3.CHO + CO_2$

(2) $COOH.CH_2.CO.\underline{COOH} \longrightarrow COOH.CH_2.CHO + CO_2$

$\underline{COOH}.CH_2.CHO \longrightarrow CH_3.CHO + CO_2$ (spontaneous)

In order to obtain information relative to this question, the Mg^{++} and the diphosphothiamine requirements of the two reactions have been determined. It was found that M. lysodeikticus requires diphosphothiamine and Mg^{++} for the decarboxylation of the carboxyl group adjacent to the carbonyl group as has been generally found with bacteria and animal tissues. On the contrary, for the decarboxylation of the carboxyl group adjacent to the methylene group only Mg^{++} is required. The difference in the coenzyme requirements indicates that the two enzymes are not alike. Whether decarboxylation of oxalacetate by yeast involves two enzymes or occurs solely by decarboxylation of the carboxyl group adjacent to carbonyl group must await further investigation. The results reported here show that with bacteria the former must be the case.

Results with acetone-treated cells made deficient with regard to diphosphothiamine and Mg^{++} are given in Table 9. Deficiencies were determined by observing the lack of ability of the preparation to oxidize pyruvate and oxalacetate aerobically. Upon addition of Mg^{++} and diphosphothiamine to the deficient cells, the oxidation of pyruvate took place (212 µl. oxygen utilized per hour with the two components supplied and only 14 µl. with no addition). The non-deficient acetone-treated cells utilized 224 µl. oxygen. When either Mg^{++} or diphosphothiamine was added

Table 9.

Pyruvate and Oxalacetate Metabolism by Mg^{++} and Diphosphothiamine

Deficient and Non-deficient Acetone Treated Cells

Substrate 0.025 M	Deficient cells +						Non-deficient acetone-treated cells
	No addition	Mg^{++}	Diphosphothiamine	Mg^{+} and diphosphothiamine	Mn^{++}	Mg^{++} and thiamine	
Pyruvate $\mu l.$ O_2 uptake	14	16	17	212	18	18	224
*oxalacetate $\mu l.$ O_2 uptake	18	20	19	219	22	22	228
*oxalacetate aerobic $\mu l.$ CO_2 evolution	41	927	38	1206	932	926	1228
*oxalacetate anaerobic $\mu l.$ CO_2 evolution	32	930	30	930	934	926	928

Total volume of reactants = 2 ml. 0.006 M $MgCl_2$; 0.006 M $MnSO_4$; 25 $\mu g.$ diphosphothiamine or thiamine. 20 mg. cells. 0.125 M PO_4 buffer pH 6.8; aerobic experiment contained air in gas space, anaerobic contained N_2.
Temperature 30.4°C. Time, one hour.
*Spontaneous decarboxylation values deducted from substrate values. With Mg^{++} 178 $\mu l.$ Without Mg^{++}, 94 $\mu l.$

separately practically no oxidation occurred. Therefore, it is apparent that both Mg^{++} and diphosphothiamine are essential for pyruvate oxidation. The addition of thiamine and Mg^{++} had no effect, which indicated that the mechanism for phosphorylating the vitamin had been destroyed by the acetone treatment.

The difference between the pyruvate and oxalacetate decarboxylations is apparent when the effects of Mg^{++} and diphosphothiamine on oxalacetate are considered. Considering oxygen uptake alone, the difference is not evident since both Mg^{++} and diphosphothiamine are required for oxygen utilization. This oxidation does not involve oxalacetate but pyruvate arising from the decarboxylation of the oxalacetate. The picture, however, is different with respect to carbon dioxide evolution. The deficient cells were unable to decarboxylate oxalacetate, e.g., only 41 µl. carbon dioxide were liberated. The addition of Mg^{++} restored both the anaerobic decarboxylation (930 µl.) and aerobic decarboxylation (927 µl.) to the original values, which were comparable to those found when both Mg^{++} and diphosphothiamine were added (930 µl. anaerobically and 1206 µl. aerobically). Obviously diphosphothiamine is not required for the decarboxylation of oxalacetate. Manganese ions can replace magnesium as a component of the decarboxylation enzyme. Mg^{++} or Mn^{++}

increased the spontaneous decarboxylation of oxalacetate and proper corrections were made.

Data given in Table 10 were obtained with the lysed deficient preparation. With this preparation it should be stated that the dehydrogenase activity had been nearly destroyed by lysis and extraction, consequently there was little oxygen uptake with pyruvate. This is in accordance with the findings of Penrose and Quastel (1930). The oxalacetate values, however show substantially the same results as those obtained with the acetone treated preparation.

These results appear to differ from those reported by Smyth (1940) and Krebs and Eggleston (1940b), who believe that thiamin is necessary for the synthesis of oxalacetate from pyruvate and carbon dioxide. Smyth working with staphylococci showed that oxalacetate can replace thiamine in the dismutation of pyruvate. He concluded that thiamine is required for the synthesis of oxalacetate which acts as a hydrogen acceptor in the dismutation of pyruvate to lactate, acetate and carbon dioxide. Therefore in the presence of oxalacetate the vitamin is not necessary for the dismutation. If this were true, the vitamin-deficient acetone preparation should oxidize pyruvate in the presence of oxalacetate but it does not since oxygen uptake and decarboxylation of pyruvate are obtained only on addition of Mg^{++} and diphosphothiamine to the oxalacetate experiments. It must

Table 10.

Metabolism of Deficient and Non-deficient Lysed Cells with Oxalacetate

Substrate 0.025 M	Deficient cells +						Non-deficient lysed cells
	No addition	Mg^{++}	Diphospho-thiamine	Mg^{++} and diphospho-thiamine	Mn^{++}	Mg^{++} and thiamine	
*oxalacetate anaerobic µl. CO_2 evolution	52	928	48	934	927	922	17
*oxalacetate aerobic µl. CO_2 evolution	54	925	49	916	922	918	920

Total volume of reactants = 2 ml. 0.006 $MgCl_2$, 0.006 M $MnSO_4$, 25 µg. diphosphothiamine or thiamine, 20 mg. cells per cup. 0.125 M PO_4 buffer pH 6.8; aerobic experiments contained air in gas space, anaerobic contained N_2.
Temperature 30.4°C. Time, one hour.
*Spontaneous decarboxylation values deducted. With Mg^{++} 178 µl. Without Mg^{++} 94 µl.

be borne in mind that with oxalacetate as the substrate, there is a sufficient supply of pyruvate at all times owing to the spontaneous decarboxylation of oxalacetate. It should be pointed out that the results of Smyth are by no means conclusive proof of the necessity for the vitamin. Although the anaerobic dissimilation of pyruvate has not been studied as thoroughly as the aerobic oxidation, it is probable that the components of the systems are similar. In the aerobic dissimilation of pyruvate (Banga et al. 1939), diphosphothiamine, a C_4 dicarboxylic acid, inorganic phosphate, adenine nucleotide and magnesium ions are components of the system. The oxidation thus requires diphosphothiamine even in the presence of C_4 acids. Certainly diphosphothiamine, in this case, has a function other than the synthesis of C_4 acids. The system investigated by Smyth is very complex and there is a possibility of a multiple deficiency. The removal of any one of these deficiencies might stimulate the reaction. The speed of the reaction may be limited by an additional factor when either thiamine or C_4 acids or both are added. Particularly in view of the results presented here, it seems that another explanation of Smyth's results may be forthcoming.

Krebs and Eggleston (1940b) investigated the dissimilation of pyruvate by pigeon liver and found an increased pyruvate disappearance in the presence of sodium bicarbonate

and carbon dioxide. They concluded that the pyruvate was used in carbon dioxide fixation, to form oxalacetate which then underwent reactions of the Krebs cycle. Thiamine was concluded to be concerned with the fixation reaction because the vitamin stimulated pyruvate dissimilation by liver from an avitaminous pigeon. This stimulation was not obtained by employing breast muscle tissue from the same bird. They concluded that the vitamin acts in a reaction occurring in the dissimilation by liver but not in that by breast muscle. Furthermore, since oxalacetate synthesis was considered to occur in liver and not in breast muscle, the vitamin was thought to act in this synthesis. The suggested function of the carbon dioxide is in accord with observations with bacteria and is attractive in view of the results of Evans and Slotin (1940) and Wood et al. (1941b)(1942) who have found that liver tissue utilizes carbon dioxide during the dissimilation of pyruvate. The evidence for the function of the vitamin in this reaction is, however, not conclusive. Krebs and Eggleston presented no proof that the breast muscle was deficient in thiamine. It can not be assumed that in an animal suffering from vitamin deficiency all of the organs are equally deficient (cf. Ochoa and Peters, 1938). Thus the liver of an avitaminous pigeon may be limited in its metabolism of pyruvate, whereas the breast muscle may still contain sufficient diphosphothiamine for an active reaction.

Therefore, the evidence offered by Krebs and Eggleston that thiamine does not function in pyruvate metabolism of breast muscle needs confirmation, since the vitamin activity will not be apparent if the muscle is not deficient, and therefore their conclusion concerning the function of the vitamin in oxalacetate synthesis may require another explanation.

Effect of heat on oxalacetate carboxylase.

It is well known that there is a heat stable component present in mammalian tissue which activates the decarboxylation of oxalacetate (Breusch, 1939). The heat stability of the newly discovered enzyme, therefore has been studied.

That this preparation is not the heat stable component is shown by Table 11. The enzyme is heat labile and is almost completely destroyed at 100°C. within five minutes. The small activity obtained under these conditions may be due to the stable component discussed by Breusch.

Irreversibility of the enzymatic decarboxylation as indicated by direct measurements.

Data were presented in Table 8 which show that oxalacetate is decarboxylated completely to pyruvate and carbon dioxide, i.e., no oxalacetate remained as determined by the aniline-citrate method. This behavior indicates that the reaction is irreversible, however inability to determine small

Table 11.

Effect of Heat on Enzyme

Substrate 0.025 M	Lysed preparation				Acetone preparation	
	Normal	60°C. 10 min.	80°C. 10 min.	100°C. 5 min.	Normal	100°C. 5 min.
*oxalacetate µl. CO_2 evolution	944	498	40	15	948	30

Total volume reactants = 2 ml. 20 mg. enzyme preparation per cup. 0.125 M PO_4 buffer pH 6.8. Gas space N_2.
Temperature 30.4°C. Time, one hour.
*Spontaneous decarboxylation values deducted.

quantities of oxalacetate by the aniline-citrate method may be responsible. The position of the equilibrium may be such that only a very small quantity of oxalacetate remains. Nevertheless, an experiment was performed in an effort to reverse the reaction. The acetone preparation was allowed to act on 0.025 M pyruvate in 0.125 M phosphate buffer, pH 7.0. The vessel was gased with a mixture of 95% nitrogen and 5% carbon dioxide. In all experiments no evidence for carbon dioxide disappearance was obtained. Oxalacetate determinations also were made and these results were likewise negative.

Physiological oxalacetate from fumarate oxidation.

In connection with the ability of the enzyme preparation to completely decarboxylate oxalacetate to pyruvate and carbon dioxide, it is interesting that when the same preparation oxidizes fumaric or malic acids, a quantity of oxalacetate, as determined by the aniline-citrate method, remains. Fumaric acid oxidation is conventionally pictured as the addition of water by the enzyme fumarase, to the double bond forming malic acid, and the oxidation of the latter acid to oxalacetic acid. The reactions are:

$$\underset{\text{fumaric acid}}{COOH.CH{:}CH.COOH} + HOH \overset{\text{fumarase}}{\rightleftharpoons} \underset{\text{malic acid}}{COOH.CH_2.CHOH.COOH}$$

$$COOH.CH_2.CHOH.COOH \xrightarrow{-2H} \underset{\text{oxalacetic acid}}{COOH.CH_2.CO.COOH}$$

Why the enzyme should not decarboxylate the oxalacetate resulting from this oxidation to completion can not be answered at this time. However, Kalckar (1939) with extracts of kidney tissue has been able to oxidize malate to phosphopyruvate. This investigator was never able to isolate phosphopyruvate, however a phosphate ester was obtained whose property to become hydrolyzed was similar to a vinyl ester (enolic ester). Lipmann (1941) proposed that the oxidation occurred by the addition of phosphoric acid to the double bond of fumaric acid to form phosphofumaric acid. The reactions are:

$$\underset{\text{fumaric acid}}{COOH.CH{:}CH.COOH} + H_3PO_4 \longrightarrow \underset{\text{phosphofumaric acid}}{COOH.CH_2.CHO(H_2PO_3).COOH}$$

$$\xrightarrow{-2H} \underset{\text{phospho-oxalacetic acid}}{COOH.CH{:}C.O(H_2PO_3).COOH} \rightleftharpoons \underset{\text{phosphopyruvic acid}}{CH_2{:}C.O(H_2PO_3).COOH} + CO_2$$

It is possible that the mechanism for the oxidation of fumaric acid as carried out by the enzyme preparation is accomplished in this manner, and that the equilibrium of the decarboxylation is such that phospho-oxalacetate accumulates in quantities large enough to detect. Structurally phospho-oxalacetate is a vinyl ester and should hydrolyze similarly to phosphopyruvate, however no phosphate fractions with such properties have been detected in the products of the fumarate oxidation by the acetone preparation. Green (1936) has shown with tissue

preparations that the oxidation product of malic acid is oxalacetic acid. He also has shown that the oxidation ceases very shortly after it starts, and that in order for the oxidation to continue, a keto fixative such as potassium cyanide or semicarbazide must be added to remove the inhibitory effect of the oxidation product. According to this author small quantities of oxalacetic acid inhibit the dehydrogenase. Peculiarly, the oxidation with the enzyme preparation from M. lysodeikticus continues, and the quantity of oxalacetate remaining does not inhibit the reaction (Table 12). The addition of oxalacetate, synthesized in the laboratory, does not inhibit the oxidation nor does the addition of a keto fixative (potassium cyanide or semicarbazide) stimulate the oxidation (Table 12). When KCN is used as the fixative, methylene blue must be substituted for the autoxidizable carrier inasmuch as the cytochrome oxidase is poisoned by KCN. An alternative explanation of this behavior is that phosphoric acid combines with the oxalacetate as an addition compound much like bisulphite complexes with keto compounds. Lipmann (1939) has suggested this type of addition to pyruvic acid during its oxidation to acetic acid, carbon dioxide and water with Lactobacillus delbrueckii. With oxalacetic acid the reaction is:

$$COOH.CH_2.CO.COOH + H_3PO_4 \longrightarrow COOH.CH.\overset{\overset{\displaystyle OH}{\cdot}}{\underset{\underset{\displaystyle O.(H_2PO_3)}{\cdot}}{C}}.COOH$$

Table 12.

Additive Effect of the Oxidation of Fumarate and of Oxalacetate by Acetone Preparation of M. lysodeikticus

Substrate 0.025 M	µl. O_2 uptake during four 15 minute intervals			
	First interval 15 min.	Second interval 30 min.	Third interval 45 min.	Fourth interval 60 min.
Fumarate	82	79	83	76
Oxalacetate	42	38	45	40
Fumarate + oxalacetate	120	114	126	122
Fumarate + semicarbazide	78	74	72	81

Total volume of reactants = 2 ml. 20 mg. cells per cup.
0.125 M PO_4 buffer pH 6.8. Gas space contained air.
0.08 M semicarbazide.
Temperature 30.4°C. Time, one hour.

This phosphorylated compound would not display any of the properties of a vinyl ester and in all probability would be a very labile substance, so that the acid conditions of the aniline-citrate method would destroy the addition complex which would behave as oxalacetic acid under these conditions. If such a reaction exists it is enzymically produced, since the addition of synthesized oxalacetic acid does not inhibit the oxidation of fumarate with M. lysodeikticus. If it were a simple non-enzymatic reaction, it would have been necessary for Green to add a keto fixative to find the oxalacetate produced by the oxidation. It is quite possible that the treatment given to his tissue preparations destroyed any enzyme which would promote the addition of phosphoric acid to carbonyl linkages. The importance of this residual "oxalacetate" which remains after fumarate oxidation will be discussed later and also evidence will be presented to show that it has properties unlike the oxalacetate synthesized in the laboratory. It will be referred to as the physiological oxalacetate. It may be stated at this time that physicochemical studies on compounds of this type would be highly advantageous. Spectrographic analyses of these labile phosphate compounds, which include the recently discovered 1,3-diphosphoglyceric acid, would be welcomed by biochemists.

Phosphate requirement for decarboxylation.

In order to investigate the phosphate requirements for the decarboxylation of oxalacetate, a phosphate deficient preparation was employed. The decarboxylation of the dibasic acid produces an alkaline reaction which interferes with the efficiency of the enzyme. Borate buffer, pH 7.2, was employed instead of phosphate with the preparation and oxalacetate, and for comparison a similar experiment with phosphate buffer was performed. The decarboxylation was permitted to continue for 40 minutes and the residual oxalacetate was determined. No oxalacetate remained in the experiments conducted with phosphate buffer, however 114 µl. oxalacetate remained in the experiments with borate buffer. 928 µl. oxalacetate was originally present. These results indicate that phosphate stimulates decarboxylation, whether it is a requirement can not be answered as yet.

Demonstration of Carbon Dioxide Fixation

General information on the use of the stable isotope of carbon.

The introduction of methods for concentration of the stable isotope of carbon with an atomic weight of thirteen, and the methods of determining relative abundances of the

isotope by means of the mass spectrometer, made it possible to investigate the reversibility of the oxalacetate decarboxylation reaction with the isotope as a tracer substance.

In order to orient the reader with regard to the use of C^{13} as a tracer in biological reactions, a few pertinent facts will be outlined. All naturally occurring substances have a normal C^{13} content of 1.09%. It is for this reason that the atomic weight of carbon is 12.01, i.e., approximately one part per hundred is C^{13}. Recently it has been shown by Swenseid et al. (1942) that some animal tissues possess lower concentrations of the isotope. Values as low as 1.06% were reported. It appears that these tissues may selectively differentiate the two forms of carbon during their metabolic activities. It is obvious that metabolic reactions can be followed conveniently with this method. Wood et al. (1941a) (1942) and Slade et al. (1941)(1942) have used the method to good advantage in their studies on the utilization of carbon dioxide by several heterotrophic forms. Mechanisms of biological reactions can be studied by first synthesizing a metabolite and then following its course of assimilation or dissimilation. If upon analysis of the products of metabolic activity one finds concentrations of C^{13} above normal, it is evident that the product was derived from the original substrate. By chemical degradation of the products,

information relative to the intermediary steps may be obtained. Precautions must be exercised, however in drawing conclusions.

Exchange reactions defined as fixation reactions.

Relative to the problem at hand, if the enzymatic decarboxylation of oxalacetate is reversible, exchange with carbon dioxide should occur during decarboxylation. Regardless of how far the equilibrium point of the reaction is to the side of decarboxylation, part of the pyruvate thus formed will be carboxylated to oxalacetate, owing to the dynamic nature of equilibria. Any exchange reaction involving carbon dioxide with the formation of a carbon to carbon linkage is considered to be fixation. Wood and Werkman have proposed the C_3 and C_1 addition hypothesis, consequently any exchange with any other than a four carbon compound will indicate the existing proposals to be incomplete.

In general the method employed was to allow the reactions to occur in the presence of $KaHC^{13}O_3$ until approximately one-half of the original substrate remained. The residual substrates were degraded and the C^{13} content of the fragments determined on the mass spectrometer.

The results of Swenseid et al. (1942) make it paramount that the standard normal complement, to which exchange results are to be compared, be determined. The source of the diethyloxalacetate used in the synthesis of the oxalacetic

acid was not known (whether of animal or mineral origin). The C^{13} values of both carboxyl groups are given in Table 13. The values 1.10% for the carboxyl group adjacent to the methylene group and 1.09% for the carboxyl adjacent to the carbonyl group are normal in accordance with determinations made on several substances by Nier and Gulbransen (1939).

Non-enzymatic exchange of $C^{13}O_2$ with oxalacetate.

Oxalacetate decarboxylates spontaneously to pyruvate and carbon dioxide. It is known that in general, deuterium exchanges with ionizable hydrogen, consequently the possibility of a non-enzymatic exchange of carbon dioxide with oxalacetate was determined. Experiments were conducted with the acid in the presence of the phosphate-bicarbonate buffer mixture but no enzyme. Results in Table 13 show that possibly there is a very slow rate of exchange with the carboxyl group adjacent to the methylene group and carbon dioxide. However, the C^{13} percentage, 1.13 with the aniline-citrate method, is only slightly above the normal of 1.09% and may lie within the limits of experimental error with the mass spectrometer ($\pm$ 2%). It should be emphasized that the time for these experiments was much longer, 210 minutes, than that of the enzymatic experiments, so as to allow approximately the same amount of decarboxylation to take place. The

Table 13.

The Exchange of Heavy Carbon Dioxide in Carboxyl Groups of Oxalacetate During Enzymatic and Non-enzymatic Decarboxylation

	C^{13} content of synthesized oxalacetate	Spontaneous decarboxylation	Enzymic decarboxylation		
			Acetone treated prep.	Mg^{++} deficient prep.	Deficient prep. + Mg^{++}
	No $C^{13}O_2$ added	Time: 210 min.	Time: 20 min.	Time: 60 min.	Time: 20 min.
	% C^{13}	% C^{13}	%C^{13}	%C^{13}	% C^{13}
*M carboxyl Aniline-citrate method of analysis	1.10	1.13 [1.12]	<u>1.23</u> [1.13]	<u>1.16</u> [1.09]	<u>1.26</u> [1.11]
*M carboxyl acid-heat method of analysis	1.10	1.12 [1.11]	<u>1.29</u> [1.12]	<u>1.16</u> [1.12]	<u>1.26</u> [1.08]
*C carboxyl $Ce(SO_4)_2$ oxidation method of analysis	1.09	1.11	1.11	1.08	1.11

Quantities in brackets represent the corresponding $NaHC^{13}O_3$ "rinse" values
*M = CO_2 from carboxyl group adjacent to methylene group
*C = CO_2 from carboxyl group adjacent to carbonyl group
Underscored figures represent C^{13} values where exchange has taken place
Original C^{13} concentration of bicarbonate added = 9%

carboxyl adjacent to the carbonyl group contains the normal complement of C^{13} to indicate no exchange had occurred.

Enzymatic exchange of $C^{13}O_2$ with oxalacetate.

Experiments conducted with the acetone preparation, oxalacetate and phosphate-bicarbonate buffer mixture show an appreciable and significant carbon dioxide exchange. The carboxyl group adjacent to the methylene group contained 1.29% C^{13} (acid heat method) a value 18.3% above the normal C^{13} complement (Table 13). The C^{13} concentration of $NaHC^{13}O_3$ rinse was 1.12% which showed definitely that the residual $NaHC^{13}O_3$ had been removed, and was not interfering with the carboxyl carbon determination. The same carboxyl group yielded 1.23% C^{13} when determined by the aniline-citrate method which agrees with the former result within experimental error.

The C^{13} concentration of the carboxyl group adjacent to the carbonyl group was 1.11% which indicates that no exchange took place in this position.

It was previously stated that Mg^{++} but not thiamine nor diphosphothiamine was required for decarboxylation of oxalacetate. The amount of exchange was determined with the Mg^{++} deficient acetone preparation, similar to the experiment outlined in the prededing paragraph. The C^{13} concentration of the carboxyl group adjacent to the methylene

group was 1.16% as determined by both the aniline-citrate and acid-heat methods of decarboxylation (Table 13). The increase above the normal complement of C^{13} may have resulted from inability to completely remove the magnesium ions by the alkaline phosphate washing. The carboxyl group adjacent to the carbonyl group contained a C^{13} concentration of 1.08% to indicate no exchange had taken place.

When Mg^{++} were supplied to the deficient preparation, exchange took place as evidenced by the 1.26% of C^{13} in the carboxyl group adjacent to the methylene group. This concentration was 15.6% above the normal complement of C^{13}, by both methods of analysis. The carboxyl group adjacent to the carbonyl group had a C^{13} concentration of 1.11% which again indicated no exchange.

Enzymatic exchange of $C^{13}O_2$ with physiological oxalacetate.

A quantity of oxalacetate remains after fumarate oxidation with the acetone preparation. This oxalacetate was referred to as physiological oxalacetate for the reason that oxalacetate as synthesized in the laboratory, is completely decarboxylated, whereas the physiologically formed acid maintains an equilibrium. Exchange experiments were conducted with this oxalacetate. Fumaric acid oxidation was carried out in the presence of $NaHC^{13}O_3$ and the residual oxalacetate was decarboxylated with aniline-citrate. The

C^{13} content of the carboxyl group was 1.47% or 34.8% above the normal complement (Table 14). The original $NaHC^{13}O_3$ in these experiments was prepared from 7% $C^{13}O_2$, whereas in all other experiments 9% $C^{13}O_2$ was employed. The amount of exchange is greater in those experiments in which physiological oxalacetate was employed, to indicate that exchange is greatly facilitated by the physiological oxalacetate.

These experiments were designed to determine the occurrence of enzymatic and non-enzymatic exchange of $C^{13}O_2$ with the carboxyl groups of oxalacetic acid. During the spontaneous decarboxylation of this acid, no appreciable exchange occurs, which indicates that the reaction is practically irreversible. If the small C^{13} concentrations indicate reversibility, the rate of the carboxylation reaction is extremely slow. Enzymically the rate of exchange is greatly enhanced and occurs exclusively in the carboxyl group adjacent to the methylene group. The enzyme evidently shifts the rate at which the equilibrium of the reaction is obtained making it possible to demonstrate the reversibility of the reaction by the exchange method. That there is no exchange in the carboxyl group adjacent to the carbonyl group is extremely interesting in view of the results of Slade et al. (1941)(1942) and Wood et al. (1942). The former working with several species of heterotrophic bacteria, and the latter with pigeon liver observed the presence of fixed carbon diox-

Table 14.

The Exchange of Heavy Carbon Dioxide in Carboxyl Groups

I	II	III	IV	V
Pyruvate carboxyl	Lactate carboxyl	Pyruvate carboxyl from lactate oxidation	α ketoglutarate *C carboxyl	Oxalacetate *M carboxyl from fumarate oxidation
Time: 90 min.	Time: 210 min.	Time: 210 min.	Time: 210 min.	Time: 60 min.
$\%C^{13}$	$\%C^{13}$	$\%C^{13}$	$\%C^{13}$	$\%C^{13}$
1.12	1.12	1.12	1.11	<u>1.47</u>

*C = carboxyl group adjacent to carbonyl group
*M = carboxyl group adjacent to methylene group
Original C^{13} concentration of original bicarbonate in column V was 7%; all others 9%
Underscored figures represent C^{13} values where exchange has taken place

ide in the carboxyl group of lactic acid. Assuming that the fixation occurred by the Wood and Werkman reaction, these authors gave two alternative explanations to account for the fixed carbon in lactic acid. (a) The reduction of oxalacetate to a symmetrical molecule, from which the lactate was eventually obtained, and (b) a suggestion by Meyerhof (1942) that the non-enzymatic shifting of OH and H in enol oxalacetate cause the carboxyl groups to lose their orientation with respect to the original carbonyl and methylene groups. The reaction may be represented as follows.

$C^{13}OOH.COH:CH.COOH \rightleftharpoons C^{13}OOH.CH:COH.COOH$

Accordingly the oxalacetate will have fixed $C^{13}O_2$ in both carboxyl groups, and the occurrence of C^{13} in the lactic acid would be easily explained. The enzymatic exchange experiments do not lend support to this mechanism since exchange was found to occur only in the carboxyl group adjacent to the methylene group. There is a possibility that the shift of the enolic hydroxyl group may be catalyzed by an enzyme not present in the acetone cells.

Since significant exchange can occur only enzymically, it is evident that exchange will occur only in those molecules of oxalacetate which are in contact with the enzyme, and which are decarboxylated and immediately carboxylated and removed from the enzyme surface. All other molecules of oxalacetate having a normal C^{13} concentration will dilute

those in which exchange has taken place. This explanation may in part account for the C^{13} concentration in the carboxyl group being less than that of the bicarbonate added. The actual degree of exchange is much greater than the results indicate.

Two possibilities may be suggested to explain the greater exchange value obtained with the physiological oxalacetate. (1) a greater proportion of the oxalacetate molecules obtained from fumarate oxidation are in intimate association with the carboxylating enzyme. This would imply that the fumarate (malate) dehydrogenase is in close proximity to the carboxylating enzyme, thereby insuring contact with the latter enzyme; (2) the oxalacetate obtained from fumarate oxidation in a different form from that synthesized in the laboratory. The latter explanation appears to be the more plausible since the synthesized oxalacetate is completely decarboxylated to pyruvate and carbon dioxide, whereas an equilibrium is established with that obtained from fumarate oxidation. The phosphate coupling reactions with such a compound have been previously discussed. Solomon et al. (1941) have shown that $C^{11}O_2$ is fixed during glycogen synthesis from lactate in rat liver. These authors have proposed the reversible reactions of the Embden-Meyerhof-Parnas scheme of carbohydrate dissimilation to account for the synthesis. Since the phosphopyruvate + adenylic acid $\longrightarrow$ pyruvate +

adenosinetriphosphate reaction in this scheme has as yet been found irreversible, they have proposed the synthesis of phosphopyruvate via the Wood and Werkman reaction. The oxalacetate is reduced to fumarate which undergoes the reactions given on Page 64. The symmetrical fumarate molecule accounts for the fixed carbon in the glycogen. This concept of carbon dioxide fixation for phosphorylation of pyruvic acid is not in accord with the occurrence of a greater exchange in the case of oxalacetate resulting from fumarate oxidation. The evidence supports the concept that the utilization reaction requires phosphate, however, the phosphorylation mechanism may be similar to that proposed by Lipmann (1939), which involves a carbonyl phosphate linkage (page 65).

General exchange during decarboxylations.

To determine whether or not exchange reactions are generally present during enzymatic decarboxylations, exchange studies were made with the following reactions; all are well known intermediate reactions in the dissimilation of glucose.

(1) $CH_3.CO.COOH + O \longrightarrow CH_3COOH + CO_2$
pyruvic acid — acetic acid

(2) $CH_3.CHOH.COOH + O \longrightarrow H_2O + CH_3.CO.COOH \xrightarrow{+O}$
lactic acid — pyruvic acid

$CH_3.COOH + CO_2$
acetic acid

(3) $COOH.CH{:}CH.COOH + O \longrightarrow COOH.CH_2.CO.COOH \longrightarrow$
fumaric acid — oxalacetic acid

$CH_3.CO.COOH + CO_2$
pyruvic acid

(4) $COOH.CH_2.CH_2.CO.COOH + O \longrightarrow COOH.CH_2.CH_2.COOH + CO_2$
α ketoglutaric acid — succinic acid

The enzymatic oxidation of pyruvate to acetic acid and carbon dioxide was carried out in the presence of $NaHC^{13}O_3$. The C^{13} concentration of the carboxyl group of pyruvate was 1.12% or practically normal within experimental error of the determination (Table 14). Since oxalacetate derived from fumarate oxidation gave higher exchange values than synthesized oxalacetate, it was considered possible that pyruvate derived from lactate oxidation might be able to bring about such an exchange. The acetone preparation oxidizes lactate to acetate and carbon dioxide. However, quantities of pyruvate accumulate which suggests that pyruvate oxidation is slower than lactate oxidation or that decarboxylation of pyruvate may also maintain an equilibrium. The oxidation of lactate was carried out in the presence of $NaHC^{13}O_3$ until determinable quantities of pyruvate accumulated (210 minutes). The C^{13} concentration of the carboxyl group of this pyruvate was 1.12% (Table 14), which indicates no exchange. The C^{13} content of the carboxyl group of the residual lactate was 1.12%, also negative with respect to exchange.

Likewise, the oxidation of α ketoglutaric acid was carried

out in the presence of the phosphate-bicarbonate mixture and the enzyme preparation. The carboxyl group adjacent to the carbonyl group in the residual α ketoglutaric acid had a C^{13} concentration of 1.11%, which is no greater than the normal complement.

The negative exchange result obtained during the oxidative decarboxylation of pyruvate is not altogether unexpected, since there was no exchange in the carboxyl group adjacent to the carbonyl group in oxalacetate. Ferdman and Epstein (1940) have reported the necessity of phosphate for lactate oxidation; consequently one would expect the pyruvate resulting from this oxidation to be phosphorylated and probably capable of being carboxylated. This, however, was not the case as no oxalacetate could be detected after lactate oxidation and in addition the pyruvate did not exhibit an ability to exchange carbon dioxide, furthering the evidence against a C_2 and C_1 addition hypotheses.

Wood et al. (1941b) have found carbon dioxide in the carboxyl group adjacent to the carbonyl group of α ketoglutaric acid formed during the dissimilation of pyruvic acid with pigeon liver. They have proposed that the carbon is initially fixed by three and one carbon addition and that the α ketoglutarate is derived from the oxalacetate. That the fixed carbon did not arise by the carboxylation of succinic acid is borne out by the negative exchange values observed in the

present investigation of the oxidation of α ketoglutarate to succinate and carbon dioxide.

CONCLUSIONS AND SUMMARY

It has been well established that the C_4 dicarboxylic acids have an important role in the respiration of mammalian tissue. Evidence that they are likewise constituents of bacterial respiration has been presented here. The function which these acids perform is not adequately understood, however working hypotheses have been proposed in the case of tissue respiration based on the Szent-Györgyi scheme and on the Krebs cycle, which may likewise be applied to bacterial respiration. In view of recent research, the hypothesis involving Krebs' postulations, to be more applicable in principle to bacteria. The most serious objection to the Szent-Györgyi scheme is that the succinic dehydrogenase must be intact for respiration, since the pathway for all hydrogen is through the fumarate $\underset{-2H}{\overset{+2H}{\rightleftharpoons}}$ succinate step.

Until recently it was believed that only the succinate dehydrogenase could transfer hydrogen to cytochrome c. It was on this hypothesis that Szent-Györgyi based his assumption that all transfer of hydrogen proceeded through succinic acid. Haas et al. (1940) have recently isolated a flavoprotein which

is capable of reducing cytochrome c. The flavoprotein acts as a transfer system between the triphosphopyridine nucleotide dependent enzymes and cytochrome c. Dewan and Green (1938) and Adler et al. (1937) have isolated a flavoprotein which oxidizes diphosphopyridine nucleotide and probably donates the hydrogen to the cytochrome system. The findings of these investigators eliminates the hypothesis that succinic acid dehydrogenase is indispensable to respiration.

Previously the work of Krebs and Eggleston (1940b) was cited, wherein they showed malonate inhibited the respiration of pigeon breast muscle and that fumarate restored the respiration proportionally to the amount of fumarate added. Clearly the malonate inhibited the succinate dehydrogenase, nevertheless respiration continued. Szent-Györgyi realized the inadequacy of his scheme insofar as the relief of the malonic acid inhibition by fumaric acid was concerned. Consequently, he postulated that fumarate has a greater affinity for the protein moiety of the enzyme than does malonate, therefore it displaces the malonate from the enzyme surface. The work of Evans and Slotin (1941) and of Wood et al. (1942) showed that respiration occurs in liver tissue poisoned with malonate, and that carbon dioxide is utilized during respiration. The fixed carbon dioxide was found in the malic, fumaric and α ketoglutaric acids. Obviously the carbon dioxide was utilized in the synthesis of

a C_4 acid which permitted respiration to continue. In M. lysodeikticus the oxidation of pyruvate, malate and fumarate is independent of the succinate system as indicated by the acetone preparation in which the succinate dehydrogenase is destroyed.

The high Q_{O_2} values obtained during the dissimilation of the C_4 dicarboxylic acids, indicate that the function of these acids involves respiration. The increased rate of respiration obtained during the oxidation of glucose when C_4 acids are added to a preparation deficient in these acids supports this view. The demonstration that these acids have a respiratory function necessitates a mechanism either for regeneration of the C_4 acids or for their synthesis. The carboxylation of pyruvate to form oxalacetate represents such a synthesis. The isolation of this enzyme sufficiently free from interfering enzymes has been effected.

The enzyme has been demonstrated by its ability to decarboxylate oxalacetate to carbon dioxide and pyruvic acid, which represents the reverse of the Wood and Werkman reaction. The reversibility of the decarboxylation has been demonstrated with the aid of the stable isotope of carbon, C^{13}. The demonstration of the reversibility of the decarboxylation reaction by direct measurements can not be made owing to the unfavorable equilibrium of the reaction. The product of carboxylation, oxalacetate, is structurally similar to

one of the reactants, pyruvate; consequently no chemical method is as yet known for the removal of the product as rapidly as it is formed.

In the presence of any enzyme capable of removing the oxalacetate, it is impossible to obtain absolute proof of the fixation reaction. For example, the point may be made that reduction of pyruvate occurs first and the carboxylation of the reduced product occurs secondly.

The dynamic nature of equilibria permits the demonstration of the reversibility of the enzymatic decarboxylation of oxalacetate with the aid of $C^{13}O_2$. If the decarboxylation is performed in the presence of $C^{13}O_2$ and permitted to continue until one-half of the original oxalacetate remains, the $C^{13}O_2$ will be found only in the carboxyl group adjacent to the methylene group of oxalacetate. This exchange occurs during the enzymatic decarboxylation but no appreciable exchange occurs during spontaneous decarboxylation.

The rate at which the equilibrium is attained may not be a factor since the enzymatic and spontaneous reactions were permitted to continue until approximately the same amount of decarboxylation had occurred. The enzymatic reaction may involve components which cannot as yet be identified. In all probability the reactants and the product of this reaction involve substrate-enzyme and product-enzyme complexes

which produce conditions unlike those in the absence of the enzyme and therefore produce new equilibrium conditions.

In contrast to the findings of Krebs and Eggleston (1940a) and Smyth (1940) who maintain that diphosphothiamine is a constituent of oxalacetate carboxylase, evidence is presented here that magnesium or manganese ions but not diphosphothiamine are essential components of the enzyme. Of interest are the results of Wood and Werkman (1940) which show that sodium fluoride inhibits the fixation reaction. It is possible that the fluoride ions precipitate the magnesium ions and thus inactivate the enzyme.

The present status of our views has been presented in the light of present knowledge. It must remain for future investigations to lead us to more complete conclusions than can be drawn at this stage of investigation.

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